REMARKS

Claims 24, 26 and 28-32 are currently pending in the application. Claim 32 are amended. The amendments find support in the specification and are discussed in the relevant sections below. No new matter is added.

Information Disclosure Statement

A supplemental Information Disclosure Statement (IDS) citing art identified in an International Search Report was filed on March 21, 2003. A copy of the 1449 form as filed is attached. Proper acknowledge of the IDS is respectfully requested.

Claim Rejection Under 35 U.S.C. §112, First Paragraph

The pending claims 24, 26, and 28-32 are rejected under 35 U.S.C. §112, First Paragraph, as alleged not being enabled. The Office Action states that "the specification, while being enabled for an *in vitro* method of determining the immune response the co-infection of mice with *M. avium* and *S. mansoni* (either with or without TNBS treatment) or the infection of mice with *T. muris* (with TNBS treatment) by determining the amount of IL-4, IL-5 and IFN-γ, does not reasonably provide enablement for a method of screening an helminthic parasite preparation for one or more components that reduce excessive Th1 immune responses, wherein said preparation is prepared by fractionating and sub-fractionating the helminthic preparation is maintained for reasons of record." The Office Action continues that "[t]he specification still does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make **and use** the invention commensurate in scope with these claims." Specifically, the Office Action states that the specification does not dsiclose "how said **sub-fraction** were used in the assay.... The specification does not provide guidance as to which biological functions (other than IL-4, IL-5 and IFN-γ production) should be tested or how the testing of said functions would result in identifying one or more components that reduce an excessive Th1 response."

First, Applicants submit that claim 24 and its dependent claims 28-30 **do not recite** a sub-fraction or the testing of a sub-fraction. Claim 26 is the only pending claim which recites "sub-fraction." Applicants submit that the specification is fully enabling for sub-fractionation

and for the assaying of sub-fractions for the presence of a biological activity that reduces an excessive Th1 immune response. However, because claims 24 and 28-30 do not recite a sub-fraction, any rejection based on an alleged lack of enablement regarding sub-fractions or their use cannot apply to claims 24 and 28-30.

Applicants submit that the specification teaches sub-fractionation at page 32, lines 7-11:

> Fractions identified as having immuno-modulatory activity in one or more of the assays may be further fractionated using chromatographic separation techniques, with subsequent assay of the sub-fractions for activity. Such sub-fractionation may be repeated at least once, or as necessary to further purify the immunomodulatory component or components of the HH [helminthic homogenate]. (Specification p. 32, lines 7-11)

Fractionation, including chromatographic fractionation is described at page 32, lines 4-6:

> "HH may be fractionated by column chromatography, HPLC, FPLC, matrix-affinity chromatography, reverse-phase chromatography, and/or other electrophoretic and chromatographic separation techniques." (Specification p. 32, lines 4-6)

With regard to the sufficiency of the disclosure related to how the sub-fractions are used in the assay, Applicants submit that iterative fractionation and testing of resulting sub-fractions for activity is a well-known and routine method for isolating the biologically active component(s) of a complex biological mixture. Applicants further submit that it is well known in the art that the same assay of biological activity can be used at each stage of a sub-fractionation procedure to monitor which fraction or fractions have the activity of interest. For example, when sub-fractionating an enzyme-containing preparation, the same enzyme assay is most often used at each stage of the sub-fractionation procedure to monitor which fraction(s) contain the activity. Exemplary references provided herewith (see Exhibits A-D) show that it was well known in the art that in fractionation procedures, the same assay for the activity of interest can be used at each iteration of the method – that is, one fractionates, assays the fractions for activity of interest, then uses fractions identified as having the activity for the next fractionation step, after which the sub-fractions are tested for the activity of interest using the same activity assay. This does not rule out the use of a different assay at any point, but it is most common that a single assay be used to monitor the purification for all steps.

In Palczewski et al. (1988, J. Biol. Chem 263: 14067-14073; Exhibit A), an iterative sub-fractionation approach is used to purify rhodopsin kinase. A retinal extract was fractionated over a DEAE cellulose column, and eluted fractions were monitored for rhodopsin kinase activity with a kinase assay. Fractions having activity in this assay are pooled and applied to a hydroxyapatite column, and eluted fractions are assayed for kinase activity using the same kinase assay.

In Soubeyrand et al. (1997, J. Biol. Chem. 272: 222-227; Exhibit B), an iterative sub-fractionation approach was used to purify a Phospholipase A2 enzyme from seminal plasma. Seminal plasma was fractionated over a butyl-Sepharose column, and a desorbed fraction was applied to a Sephacryl S-300 sieving column, after which fractions were tested for Phospholipase A2 activity. Fractions containing the activity were applied to a Q-Sepharose column and fractions were tested for Phospholipase A2 activity using the same assay method.

In Ostergaard et al. (1997, J. Biol. Chem. 272: 30009-30016; Exhibit C), an iterative sub-fractionation approach was used to purify an L-Galactono-γ-Lactone Dehydrogenase (GLDase) from cauliflower plants. A crude cauliflower mitochondrial extract was passed over a DEAE-Sepharose column, and eluted fractions were assayed for GLDase by monitoring the reduction of Cytochrome *c*. Fractions having activity in this assay were passed over a phenyl-Sepharose CL-4B column, and eluted sub-fractions were assayed for GLDase activity using the same assay. The active sub-fractions were pooled and applied to a Sephacryl SF-200 gel filtration column, and the sub-fractions were monitored for activity using the same GLDase assay. Subsequent sub-fractionation steps included two different anion exchange FPLC sub-fractionations and an HPLC sub-fractionation, with GLDase activity monitoring of the sub-fractions using the same assay at each stage.

In Ma et al. (1993, J. Biol. Chem. 268: 20360-20365, Exhibit D), an iterative sub-fractionation approach was used to purify an Electron-transfer Flavoprotein:Rhodoquinone Oxidoreductase (ETF-RO) from anaerobic mitochondria of the parasitic nematode Ascaris suum. A precipitated and re-olubilized protein preparation from mitochondria isolated from adult Ascaris suum muscle was applied to a Sephadex G-100 column, and a sub-fraction was applied

to a DEAE-Bio-Gel column. Eluted sub-fractions were assayed for ETF-RO activity using a spectrophotometric assay for the reduction 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone (DPB). Further sub-fractionation was performed by hydroxyapatite column chromatography. Activity in the sub-fractions was monitored with the same ETF-RO assay.

In view of the above, which provides only but a few of the many published references using an iterative sub-fractionation approach, Applicants submit that it was well known in the art at the time of the invention that one can assay a sub-fraction of a homogenate for the presence of a biological activity of interest using the same assay used to determine the activity in preceding fractions. Thus, applicants submit that there is no need for the specification to specify "how said sub-fractions were used in the [claimed] assay." One of skill in the art would know that any of the assays provided by the specification can be used to test not only fractions, but also sub-fractions of a helminthic parasite homogenate for the presence of a biological activity that reduces a Th1 immune response. That is, one of skill in the art would know that the same assay can be used for fractions and sub-fractions. Thus, Applicants submit that enablement does not require the specification to specify which assay is to be used with sub-fractions.

The specification provides guidance on assays and determination of a Th1 response at a number of places, for example, page 21, line 21 to page 24, line 22 and in the Examples. Specifically, at page 22, line 3-4, IFN-γ, TNF-α and IgG2a are taught to characterize a Th1 response, whereas IL-4, IL-5, IgE and IgG1 are taught to typify a Th2 reaction. At page 22, lines 4-10 the specification teaches that serum can be assayed for cytokine and immunoglobulin concentrations, and that flow cytometry can be used to examine Fcγ3 expression on macrophages (Th1) and MHC Class II expression on B cells (Th2). Assays for the detection of cytokines by flow cytometry and ELISA are further described at page 22, line 17 to page 23, line 20. Isotype-specific immunoglobulin assays are described at page 23,line 21 to page 24, line 2. Additional assays to determine lymphocyte secretion of antibodies or cytokines are described at page 24, lines 3-26.

The Office Action states that "the specification does not provide guidance as to which biological functions (other than IL-4, IL-5 and IFN-γ production) should be tested." Applicants

respectfully disagree. First, as noted above, the specification teaches that TNF-α and IgG2a, in addition to IFN-γ, are indicative of a Th1 immune response. These cytokines can be monitored according to assays known in the art and described in the specification as noted above. Second, Applicants submit that it is not necessary for the specification to describe more than one assay that would function to measure a Th1 immune response in order to be enabling. As stated by the Federal Circuit in *Engel Industries, Inc. v. Lockformer Co.* (946 F.2d 1526, 20 U.S.P.Q.2d 1300 (Fed. Cir. 1991)), "[t]he enablement requirement is met if the description enables any mode of making and using the claimed invention" (at 1304). Thus, the description of assays for IFN-γ, TNF-α and IgG2a are sufficient to meet the enablement requirement with regard to the determination of a Th1 immune response as required by the claims.

The Office Action states that the specification does not provide guidance on "how the testing of said functions [referring to IFN-γ, IL-4 and IL-5 production] would result in identifying 'one or more components that reduce an excessive Th1 response.'" Applicants respectfully disagree. The specification teaches at page 32, lines 9 to 11 that "[s]uch sub-fractionation may be repeated at least once, or as necessary to further *purify the immunomodulatory component or components* of the HH" (emphasis added). That is, the purified immunomodulatory component(s) identified by the fractionation approach will result in identifying one or more components that reduce an excessive Th1 immune response.

The Office Action states that "the specification is silent on how one would perform the 'assay' step of the claimed methods *in vivo*." The Action continues "The specification provides no guidance on what parameters or markers are measured, how said parameters or markers are measured or even how samples are obtained." The Action also states that the working examples do not apply to the instant invention because "they exemplify the use of intact ova not the in vivo screening of helminthic fractions or sub-fractions." The Office Action concludes that "given the total lack of guidance provided by the specification showing sub-fractionation or preparations and how one would perform the claimed method in vivo, it would require undue experimentation by one of skill in the art to make and use the invention commensurate in scope with the claimed subject matter." Applicants respectfully disagree.

First, as discussed above, Applicants note that a "sub-fraction" or "sub-fractionation" is not recited in the independent claim, claim 24. Thus, any basis of rejection hinging on sub-fractionation cannot apply to claim 24. Also as discussed above, the specification *does* teach sub-fractionation of preparations and testing of those sub fractions; to the extent that the enablement rejection is based upon a "total lack" of such teaching, Applicants request reconsideration and withdrawal of the rejection.

Second, with regard to assays performed *in vivo*, the specification provides guidance at page 30, line 23 to page 34, line 20, and in the Examples. More specifically, the specification teaches specific animal models for assaying the effect of a HH fraction or sub-fraction on an excessive Th1 immune response in Table 1 (page 33-34), including animal models for Inflammatory Bowel Disease, Rheumatoid Arthritis, Insulin-Dependent Diabetes (type 1), Lupus , sarcoidosis, Multiple Sclerosis (MS), Autoimmune Thyroiditis, colon polyps/colon cancer and allergic airway diseases.

Example 8 provides details of the use of the mouse EAE model of MS to investigate the effect of schistosomes on autoimmune disease. The specification teaches in Example 8, page 48, lines 14-16 that "Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) involving autoimmune CD4+ helper T cells, particularly cells of the T helper 1 (Th1) subgroup." *That is, the specification teaches that MS involves an excessive Th1 immune response.* The use of the murine EAE mouse model of MS to investigate the effect of helminthic preparations on the Th1 response is also described at page 31, lines 4-8, which state that "Helminthic homogenate can be compounded with myelin basic protein (MBP) or PLP139-151 and mixed with CFA (adjuvant). SJL/J mice immunized with HH/MBP will develop no or greatly attenuated experimental autoimmune encephalomyelitis (EAE)." The parameters measured in Example 8 are described in the specification at page 28, lines 5, in the section titled "Multiple Sclerosis – Evaluation of Inflammation." This section describes criteria for clinical assessment of MS symptoms in the EAE mouse *in vivo* model. The criteria include motor function assessment, brain and spinal cord histology, and analysis of dispersed splenocytes and cells from other regions. Applicants submit that this is an *in vivo* assay for the effect of helminthic components on an excessive Th1 response. A difference in the listed clinical criteria

in animals given a fraction of a helminthic homogenate is indicative of an effect against an excessive Th1 immune response.

Also with regard to guidance on *in vivo* assays and parameters to monitor, Example 3 provides details of the murine TNBS colitis *in vivo* model and its use to investigate the effect of schistosome infection on an excessive Th1 immune response. The Example provides guidance regarding measurement of a Th1 response in mesenteric lymph nodes and spleen cells in response to T cell stimulation with anti-CD3 antibody (see page 40, lines 25-27, Table 2 (page 41) and Table 3 (page 42). Further guidance with regard to *in vivo* parameters to monitor is provided in Example 3 on page 42 where intestinal inflammation parameters are assessed on a 4 point scale – the scale is described at page 38, lines 309 in the section titled "Evaluation of Mucosal Inflammation." Applicants submit that this model is set forth with sufficient clarity to permit one of skill in the art to use it to monitor the effect of a fraction or sub-fraction of a helminthic homogenate.

The Action points out that the working examples (e.g., Example 8, Example 3) use injection of intact ova, rather than a fraction or sub-fraction, arguing that these examples therefore do not apply to the instant invention. However, the specification teaches the use of fractions of helminthic homogenate in the EAE *in vivo* model and other models in the paragraph at page 31, lines 4-13. Specifically, lines 4-5 state "Other *in vivo* models exist that are useful for assaying helminthic homogenate or ***fractions of such homogenate*** for the ability to modulate an immune response" (emphasis added). Specific *in vivo* models referred to in this paragraph include the EAE model discussed above and described in Example 8, the IL-10 -/- and TNBS treatment models for IBD (described in Example 3), and the NOD type 1 diabetes mouse model. The last sentence of this paragraph states that "Injections of HH may prevent any of the autoimmune or excessive inflammatory diseases listed in Table 1." That is, one would assay the effect of a fraction or sub-fraction of HH by injecting it into an animal that models disease and monitoring symptoms.

Also with regard to the alleged lack of teaching of how a fraction or sub-fraction is used in an *in vivo* assay, the specification specifically addresses component vaccines and their use in

the section titled "Non-viable Component Vaccines" at page 17, line 23 to page 19, line 11. Specifically, the specification states at page 19, lines 7-11 that

> "Component vaccines can also be developed from larvae and adult worms of helminthic parasites. Larvae or worms are isolated from preparatory animals grown in SFP conditions. Vaccines that employ non-viable intact organisms or proteins, lipids or carbohydrates isolated from the helminth *are prepared and utilized in a manner similar to that previously described for helminth eggs.*" (Emphasis added)

In view of this teaching that components are administered in a similar manner as helminth eggs, i.e., intact ova, Applicants submit that the teachings of Examples 8 and 3 are relevant to the use of *in vivo* assays for reduced Th1 immune responses in *in vivo* models. That is, one would perform the assays using components (fractions, sub-fractions) in the same manner as taught for ova in Example 3 or Example 8. In view of this and the preceding discussion relating to the other points raised in the Office Action, Applicants submit that *in vivo* assays for assaying a fraction of a helminthic parasite homogenate for the presence of a biological activity that reduces an excessive Th1 immune response are fully enabled, and respectfully request reconsideration and withdrawal of the enablement rejection of claims 24, 26 and 28-32.

Rejection under 35 U.S.C. §112, Second Paragraph:

Claim 32 is rejected as indefinite under 35 U.S.C. §112, second paragraph.

The Office Action states that claim 32 is indefinite because it is unclear how one would assay activity *in vivo*. The Office Action states that the claim fails to identify "what, if any, assays would be considered an '*in vivo assay*' to detect a reduction in an excessive Th1 immune response," and that it also fails to recite the active steps required in order to fulfill the stated objective of the method claim. The Office Action continues to state that the working examples cited do not apply to the instant invention as they exemplify the use of intact ova not the *in vivo* screening of helminthic fractions or subfractions because all the disclosed assays were performed *in vitro*.

Applicants submit that the amendment of claim 32 herein is sufficient to overcome this rejection. Specifically, claim 32 as amended recites "wherein said assaying comprises

administering a fraction from step (c) to a mammal and detecting a Th1 response in said mammal." Applicants respectfully request reconsideration of the claim.

Rejection under §103(a) over Pearce et al. (1991) in view of Pearce et al. (1988):

Claims 24, 26 and 28-32 are rejected under 35 U.S.C. §103(a) as obvious over Pearce et al. (J. Exp. Med. 173: 159-166, 1991) in view of Pearce et al. (P.N.A.S. 85: 5678-5682, 1988).

The Advisory Action states that the method disclosed by Pearce et al. (1991) "differs from the claimed invention in that they do not explicitly disclose a method of preparing an helminthic parasite antigen comprising homogenizing, separating homogenate fractions and identifying sub-fractions for biological activity," but that Pearce et al. (1988) disclose a method of preparing antigens from *Schistosoma mansoni* that comprises obtaining adult schistosomes, homogenizing in phosphate buffered saline, centrifuging and purifying by immunoaffinity chromatography. The Office action states that it would have been obvious to prepare *Schistosoma* antigens utilizing the homogenization and immunoaffinity column chromatography disclosed by Pearce et al. (1988) and assay the resulting fractions for the ability to reduce excessive Th1 responses utilizing the assay methods disclosed by Pearce et al. (1991). The Office Action concludes that it would have been expected, barring evidence to the contrary, that the purified *Schistosoma* antigens would be identified for their ability to reduce excessive Th1 responses because Pearce et al. (1991) specifically identify and compare antigens and their abilities to down regulate Th1 cytokine production. Applicants respectfully disagree.

Applicants submit that Pearce et al. (1991) provides no motivation to screen a helminthic parasite preparation for one or more components that reduce an excessive Th1 immune response, because the reference focuses on the beneficial aspects of stimulating Th1 function. The Abstract states "the data suggest that coincident with the induction of Th2 responses, murine schistosome infection results in an inhibition of **potentially protective Th1 function**." (Emphasis added.) The reference also states "[t]hus, taken together, these previous studies suggest that infection stimulates a non-protective Th2-type response while vaccination induces protective Th1 cells" (p. 160, left column, first paragraph). Because the reference specifically

refers to the Th1 response as "protective," there is no reason that one of skill in the art would be motivated screen a helminthic parasite preparation for one or more components that *reduce* a Th1 response, as in the claimed invention.

The Pearce et al. 1991 reference teaches that "[i]n the mouse, infection with *Schistosoma mansoni* results in an egg-producing infection and associated **disease**, whereas vaccination with attenuated larval stages produces a substantial and specific **immunity** in the **absence of egg-induced pathology**." (Abstract; emphasis added.) That is, egg-producing infection causes disease, and vaccination with attenuated larval stages provides protection from such infection, without the disease. Because the Th1 response described in the reference is associated with protective immunity from disease causing infection, one would not be motivated to screen for a component of a helminthic parasite preparation that reduces a Th1 response as claimed.

The Office Action states:

> "Applicant is reminded that the instant claims are drawn to a method of screening a helminthic preparation for one or more components that reduce an excessive Th1 immune response. The method comprises preparing and fractionating the preparation and assaying the products for the ability to reduce an excessive Th1 immune response. *They are not drawn to methods of reducing a Th1 immune response.*" (Emphasis added)

Applicants submit that the fact that the claims are drawn to screening assays and not to methods of reducing an excessive Th1 response is irrelevant to the alleged obviousness of the claimed invention over the Pearce et al. references. The *context* of the references must still be considered in arriving at any motivation to combine the teachings of the references. There is no recognition in either Pearce et al. reference that a Th1 immune response is something to be avoided, so there is no reason that one skilled in the art, cognizant of the two Pearce et al. references, would screen a helminthic parasite preparation for one or more components that reduce a Th1 response as claimed. Further, there is no teaching of an "excessive" Th1 immune response in either reference, so there is no reason one would screen for a *Schistosoma mansoni* component that would reduce such a response.

Applicants submit that the Pearce et al. (1988) reference cited does not alter the understanding of the Pearce et al. (1991) reference regarding the protective role of the Th1

response in immunity to *Schistosoma mansoni* infection. Thus, not only would one of skill in the art not have been motivated by the teachings of Pearce et al. (1991) to screen a helminthic parasite preparation for one or more components that reduce an excessive Th1 immune response as claimed, no combination of that reference with the Pearce et al. (1988) reference would suggest such a method. As such, Applicants submit that the claimed invention is not obvious over the combination of these references. Applicants respectfully request the withdrawal of the §103(a) rejection of claims 24, 26 and 28-32 over this combination of references.

Applicant submits that all pending claims (i.e., claims 24, 26, and 28-32) are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney/agent of record.

Respectfully submitted,

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EXHIBIT A

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Purification and Characterization of Rhodopsin Kinase*

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Rhodopsin kinase was purified to near homogeneity by affinity binding to light-exposed rod cell outer segment membranes, followed by DEAE-cellulose and hydroxyapatite chromatography. This resulted in a 1055-fold purification of highly active rhodopsin kinase with an overall recovery of 19%. Rhodopsin kinase is a single polypeptide chain with M_r = 67,000–70,000 as determined by gel filtration and SDS-PAGE.

The kinetic parameters of the enzyme for freshly bleached rhodopsin are K_m = 4 μM and V_{max} = 700 nm l/min/mg whereas for ATP K_m = 2 μM (which is a low value for kinases generally, and about 20 times l wer than comparable measurements for a kinase of a similar type, the β-adrenergic-receptor kinase (Benovic, J. L., Mayor, F. Jr., Staniszewski, C., Lefkowitz, R. J., and Caron, M. G. (1987) *J. Biol. Chem.* 262, 9026–9032). GTP, on the other hand, is a very poor substrate (K_m = 1 mM, V_{max} = 10 nmol/min/mg). Rhodopsin kinase is competitively inhibited by adenosine and its mono- and diphosphate derivatives, but not by most other adenosine derivatives. Based upon measur ments with 28 nucleotide derivatives, the ATP-binding site of rhodopsin kinase appears to have more specific requirements than that for other kinases.

Compounds such as cGMP, inositol trisphosphate, and others that change concentration during exposure of rod cells to light have only minor inhibitory effects on the kinase activity, with the exception of inositol monophosphate, which can activate the kinase about 20% at 50–100 μM. Rhodopsin kinase has been difficult to store with retention of activity, but can be successfully stored frozen at −20 °C in 20% adonitol.

Rhodopsin is the best characterized member of a family of homologous receptors that operate via G-proteins (the adrenergic receptors, muscarinic acetylcholine receptor, etc. (1)). As part of their functions as signal transducers, the receptors become phosphorylated by protein kinase(s) in a reaction believed to deactivate the receptor. Rhodopsin kinase is the enzyme which phosphorylates light-exposed rhodopsin in rod cell outer segments. We have purified rhodopsin kinase from retinal rod cells in order to better study its enzymatic properties and role in receptor inactivation.

Rhodopsin kinase was first detected when retinal rod outer segments were incubated with [^{32}P]ATP in the presence of light, resulting in the incorporation of ^{32}P into rhodopsin (2–4). The phosphorylation of rhodopsin has since been shown to occur *in vivo* (5). The reaction has been studied in intact whole retinas (6), homogenized retinas (7), rod outer segments (8), and with rhodopsin kinase which has been submitted to one or more steps of purification (9–12). Many properties of the enzyme have been elucidated (see Ref. 13), but the kinase has not been obtained in purified form or studied in the detail available for many other kinases (14). A purification procedure for rhodopsin kinase was published recently (15). However, the specific activity of the rhodopsin kinase preparation (10 nmol of phosphate/min/mg) is considerably less than we report here (960 nmol/min/mg). Its molecular mass is a matter of disagreement, with reports ranging from M_r = 52,000 (10) to M_r = 79,000 (11). Although many workers have reported that rhodopsin kinase activity is not influenced by cyclic nucleotides (16–18), there are reports to the contrary (19–21). A number of substrate analogues have been tested for their influence on rhodopsin kinase activity, but a systematic study of the requirements of rhodopsin kinase's ATP binding site has not been undertaken. Such studies are best performed on the purified enzyme.

An enzyme which appears to be similar to rhodopsin kinase, has been shown to phosphorylate the ligand-bound form of the β- and α_2-adrenergic receptors (22, 23). This enzyme, β-adrenergic receptor kinase, phosphorylates the β-adrenergic receptor leading to homologous receptor desensitization (24, 25). Rhodopsin kinase is able to phosphorylate the β-adrenergic receptor (26), thus indicating that not only are the receptors members of a related family, but that these two kinases may be members of a kinase family specific for receptors which operate via G-proteins. A kinase of this type may also be responsible for phosphorylation of the muscarinic acetylcholine receptor (27). In this investigation we report that the purified rhodopsin kinase shares many of the properties of the β-adrenergic receptor kinase.

MATERIALS AND METHODS

Isolation of Rod Outer Segments—Rod outer segments (ROS)[1] were prepared from fresh bovine retinas obtained locally, or from frozen retinas (Lawson, Inc., Omaha, NE). We followed the procedure of Schnetkamp *et al.* (28) for the preparation of intact rod outer segments and the procedure of Wilden and Kühn (8) for preparation of membrane-bound rhodopsin to use as a substrate for rhodopsin kinase. ROS were also prepared from frozen retinas under normal room illumination but otherwise following the procedure of Wilden

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This work is dedicated to the memory of our colleague and friend, Hermann Kühn.

¶ Supported by a Jules and Doris Stein Professorship from Research to Prevent Blindness, Inc.

[1] The abbreviations used are: ROS, rod cell outer segments; DTT, dithiothreitol; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate; HPLC, high performance liquid chromatography; $Mg(OAc)_2$, magnesium acetate.

and Kühn (8). These preparations yielded as much rhodopsin kinase activity as that obtained from ROS prepared from fresh retinas in dim red light by the method of Schnetkamp. ROS preparation and all kinase separation steps were carried out at 4 °C.

Extraction of Rhodopsin Kinase Activity from Rod Outer Segments—ROS from 400 retinas were suspended in 400 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM $Mg(OAc)_2$, 5 mM DTT, and 30 μg/ml each of the protease inhibitors aprotinin, benzamidine, leupeptin, and pepstatin. The suspension was homogenized with a glass-glass homogenizer and then illuminated at 0 °C for 2 h with a 150-watt floodlamp approximately 20 cm from the sample. The ROS membranes were then collected by centrifugation at 16,000 × *g* for 30 min at 4 °C. The supernatant from this centrifugation is referred to as the "Mg extract" and contains the other kinase activities found in ROS (*e.g.*, cAMP-dependent kinase and protein kinase C. We have been able to prepare protein kinase C from this extract as previously described by Kelleher and Johnson (29)). The ROS membrane pellets were then resuspended in 100 ml of 20 mM Tris-HCl (pH 7.5) containing 10 mM DTT, 60 mM KCl, and 20 μg/ml each of aprotinin, benzamidine, leupeptin, and pepstatin. The suspension was homogenized with a glass-glass homogenizer and then stirred for 12 h in the dark at 4 °C. The supernatant from this extract, referred to as the "KCl extract," was collected after centrifugation at 16,000 × *g* for 45 min at 4 °C. If kinase yields of less than 70–75% were obtained, the KCl extraction step was repeated. If required, the supernatant was recentrifuged in order to remove all ROS membranes.

DEAE-cellulose Chromatography of Rhodopsin Kinase—The pH of the above KCl extract was adjusted to 7.8 (by addition of 10% Tris solution) and the extract was diluted to a concentration of 50 mM KCl before applying to a 1.6 × 20-cm column of DEAE-cellulose (Whatman DE52) which had been equilibrated with 75 mM Tris-HCl buffer containing 1 mM $Mg(OAc)_2$ and 1 mM DTT. Fractions of 2 ml were collected from the column at a flow rate of 12 ml/h. The column was monitored at 280 and 225 nm, and for kinase activity using 50 μl of each fraction in the kinase assay described below. After loading, the column was washed with equilibrating buffer until the $A_{280\,nm}$ or $A_{225\,nm}$ returned to baseline. Then a KCl gradient (250 ml total, from 0 to 0.25 M KCl, in the same buffer) was used to elute rhodopsin kinase.

Hydroxyapatite Chromatography of Rhodopsin Kinase—A hydroxyapatite column (1.0 × 10 cm) was prepared and equilibrated with 75 mM Tris-HCl buffer (pH 7.8) containing 1 mM $Mg(OAc)_2$ and 1 mM DTT. Pooled fractions containing rhodopsin kinase from the DEAE-cellulose chromatography were loaded directly onto the hydroxyapatite column, and the column was washed with equilibrating buffer at a flow rate of 10 ml/h until the $A_{220\,nm}$ returned to baseline. The column was then eluted with equilibrating buffer containing 0.4 M KCl. 2-ml fractions were collected. Aliquots (50 μl) of the fractions were used to assay for rhodopsin kinase activity as described below. Fractions containing kinase activity were pooled.

Protein Determinations—Protein concentrations were measured using the micro-Bradford method (30) with bovine serum albumin as the standard.

SDS-PAGE—Protein solutions were desalted using a Nucleosil C_4 HPLC column (32), and then SDS-PAGE was performed according to Laemmli (31) with 12% acrylamide gels in a Hoeffer minigel apparatus.

Molecular Weight Determined by Gel Filtration—Gel filtration on Sepharose CL 6B-200 was used to determine the molecular weight of the native kinase. A 1.6 × 100-cm column (Pharmacia LKB Biotechnology Inc.) was prepared using gel equilibrated with 67 mM potassium phosphate (pH 7.0) which contained 0.1 mM EDTA, 1 mM $Mg(OAc)_2$, and 5 mM DTT. The column was developed with this buffer at a flow rate of 10 ml/h, and 2.2-ml fractions were collected. Molecular weight standards used were aldolase (160,000), bovine serum albumin (67,000), ovalbumin (43,000) carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000) with blue dextran included to indicate the void volume and sodium azide to mark the included volume. For standardization, the column was monitored by absorbance at 280 or 220 nm. For molecular weight determination of rhodopsin kinase, the KCl extract from 30 retinas containing about 5 μg of rhodopsin kinase in 3 ml of extraction buffer (20 mM Tris-HCl, pH 7.5, containing 10 mM DTT and 60 mM KCl) was submitted to gel filtration on the standardized column. The column was monitored for kinase activity by withdrawing an aliquot of each fraction for the kinase assay.

Assay for Rhodopsin Kinase Activity—All rhodopsin kinase assays were performed using rhodopsin in urea-washed ROS membrane as substrate (13). Briefly, rhodopsin kinase was incubated under illumination at 25 °C with 10 μM rhodopsin, 100 μM [γ-^{32}P]ATP (1–3 × 10^4 cpm/nmol) in 75 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT and 1 mM $Mg(OAc)_2$. The reaction was stopped with 10% trichloroacetic acid solution containing 10 mM H_3PO_4. Rhodopsin-containing membranes were collected by centrifugation and the pellet was washed repeatedly with the trichloroacetic acid/H_3PO_4 solution to remove unbound ^{32}P. Concentrations of effectors were determined spectrophotometrically using extinction coefficients as described (33, 34). Inhibition constants were obtained by nonlinear regression analysis and averaging of results from at least two separate experiments. Concentrations of inhibitors (Sigma) were in the range of ~0.2–8 × K_m. All experiments for determination of K_i were performed with rhodopsin kinase prepared on a small scale (100 retinas) through the DEAE-cellulose chromatography step.

Stability of Rhodopsin Kinase—Activity of rhodopsin kinase was determined under standard conditions following freezing (in liquid nitrogen or at −20 °C) or exposure to defined temperatures for 2 h. The effect of the following polyalcohols or carbohydrates was examined: adonitol, sucrose, α-methylmannopyranose, *myo*-inositol, sorbitol, erythritol, mannose, maltose, glucose, fructose, and glycerol. Compounds were used at a concentration of 20%, except for *myo*-inositol which was used at 90% of saturation.

RESULTS

Molecular Weight of Rhodopsin Kinase by Gel Filtration—The molecular weight of the protein kinase which phosphorylates bleached rhodopsin was determined as shown in Fig. 1. We find a major peak with M_r = 67,000 (in agreement with an earlier report (35)). This is consistent with the molecular weight observed on SDS-PAGE after purification of the kinase to homogeneity (M_r = 70,000 protein band). This indicates that the kinase is composed of a single polypeptide chain and contains no subunit structure. A minor peak of activity was observed eluting at a molecular weight of about 50,000. This component makes up about 10% of the activity in this preparation (and about 5% in another preparation which was examined). This minor component may be a product of proteolysis of the 67,000 protein (see "Discussion").

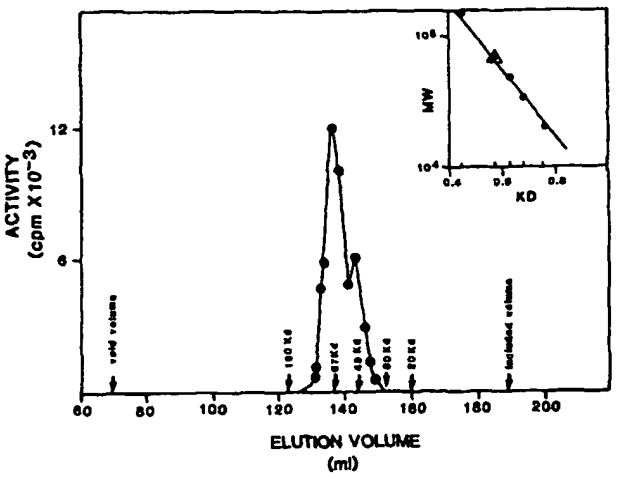

FIG. 1. **Determination of the molecular weight of rhodopsin kinase by gel filtration.** Rhodopsin kinase, partially purified by affinity binding to bleached ROS membranes, was submitted to gel filtration on a standardized Sepharose CL-6B column as described under "Materials and Methods." Elution positions of the molecular weight standards are indicated by *arrows*. Kinase activity was monitored by assaying aliquots of each fraction for ability to phosphorylate bleached ROS membranes. The counts incorporated into rhodopsin in this assay are plotted *versus* elution volume. In the *inset*, log (*MW*) of the standards is plotted *versus* kilodalton (*KD*) with the *open triangle* indicating the molecular weight of the major peak of kinase activity at 67,000. A minor peak of activity is observed at 50,000. The same results were obtained in duplicate experiments.

TABLE I

Purification of rhodopsin kinase

Rhodopsin kinase was purified as described under "Materials and Methods." The standard procedure used 400 retinas. Protein concentration was determined by the micro-Bradform method (30). Kinase activity was measured at 25 °C using urea-washed rod outer segments and [γ-^{32}P]ATP as substrates. The table summarizes results from nine preparations.

Purification step	Volume	Protein	Activity	Specific activity	Purification		Yield	
					Step	Overall	Step	Overall
	ml	*mg*	*nmol P_i/min*	*nmol P_i/min/mg protein*	*-fold*		*%*	
Rod outer segments	400	280 ± 28	255 ± 32	0.91	1	1	100	100
Rod outer segments after Mg extract	110	231 ± 18	264 ± 40	1.14	1.25	1.15	103	103
Mg extract	380	52 ± 11	18 ± 6	0.35				
KCl extract	100	22 ± 5	168 ± 60	7.6	83	83	66	66
DE-cellulose 52	50	1.5 ± 1	92 ± 40	61	8	100	54	36
Hydroxyapatite	15	0.05 ± 0.016	48 ± 11	960	15	1055	52	19

Purification of Rhodopsin Kinase—The first step in obtaining a pure rhodopsin kinase preparation is to extract the kinase from the retina or outer segments. We found that when rhodopsin kinase was prepared from sealed ROS made by the Schnetkamp method (28), or from ROS prepared from retinas under room lights, approximately twice the yield of kinase was obtained compared with that from ROS prepared by standard methods. The extraction step of our kinase preparation was based on the protocol of Sitaramayya (12) with several modifications which include shorter times for the extractions. More Mg^{2+} (10 mM) was included in the Mg^{2+} extracting buffer in order to improve binding of kinase to rhodopsin. Salt concentration was reduced in the KCl extraction buffer in order to achieve better binding of kinase to the ion exchange resin in the next purification step. The KCl extraction was performed in the dark in order to facilitate dissociation of the light-induced binding of the rhodopsin kinase to rhodopsin. Essentially all of the rhodopsin kinase activity remains with the membrane pellet when 10 mM Mg^{2+} is used (Table I), whereas in the presence of 1 mM Mg^{2+}, from 20–40% activity was lost at this step. This extraction step also appears to achieve a separation of rhodopsin kinase from the other protein kinase activities in the ROS. Extraction of the pellet with approximately isomotic KCl-Tris buffer in the dark yielded about 70% of rhodopsin kinase activity in the supernatant.[2]

By using DEAE-cellulose (Whatman DE52), kinase was bound to the resin and eluted at low ionic strength with a yield of 54% (Fig. 2 and Table I). DEAE-Sephacel (Pharmacia) can be substituted for DE52 (Whatman), but no rhodopsin kinase activity could be recovered from DEAE-cellulose (Sigma).

When the rhodopsin kinase peak was pooled and loaded on the hydroxyapatite column, the rhodopsin kinase activity was bound to the column and 52% was recovered upon elution using a step gradient (Fig. 3 and Table I). SDS-PAGE indicated the presence of essentially one protein with a M_r = 70,000 in the rhodopsin kinase activity pool from this hydroxyapatite column. The hydroxyapatite column gave much better resolution of rhodopsin kinase using a gradient with KCl rather than with potassium phosphate. This behavior is typical for a basic protein (36).

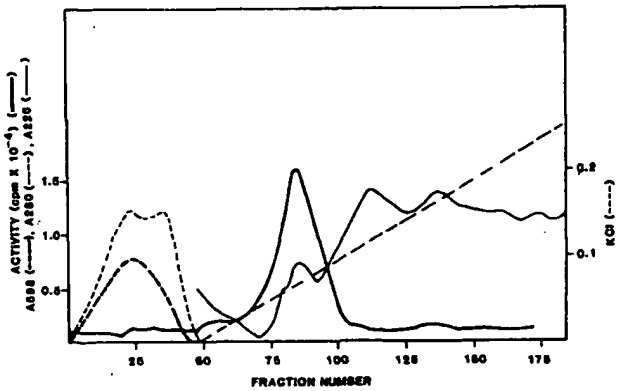

FIG. 2. **DEAE-cellulose chromatography of rhodopsin kinase.** The KCl extract from 400 bovine retinas was adjusted to pH 7.8 and then applied to the DEAE-cellulose column (1.6 × 20 cm) equilibrated with 75 mM Tris-HCl buffer (pH 7.8) containing 1 mM DTT and 1 mM $Mg(OAc)_2$. The column was washed until the absorbance of the effluent at 280 nm fell below 0.05. The elution was carried out with a linear KCl gradient (2 × 125 ml of 0–0.25 M) in the same buffer. 2.0-ml fractions were collected. The protein content was determined by the micro-Bradford method (30).

Stability of Rhodopsin Kinase—It has proven difficult to store rhodopsin kinase with retention of activity. Shichi and Somers (10) reported that at 3 °C, 50% of the activity was lost in a week. They found that K^+, NH_4^+, and glycerol stabilized the activity somewhat. Many investigators have found that crude extracts lost their activity in a few days when stored in the cold. Sitaramayya (12) found that without protease inhibitors, 90% of the activity was lost in 4–5 days whereas in the presence of protease inhibitors, 76% of the original activity remained after 37 days. We find that the half-life of our KCl extract is about 30 days when stored at 4 °C. The DE-cellulose preparation of rhodopsin kinase has a half-life of about 7–15 days, and highly purified kinase has a half-life of about 3–5 days. Dilution of the purified rhodopsin kinase is also deleterious to kinase activity speeding the decay of the activity. In a similar system, Benovic *et al.* (22) observed a half-life of the β-adrenergic receptor kinase of 5–10 days. In addition, we have observed a very marked sensitivity of rhodopsin kinase to organic solvents (*e.g.* an incubation of only 2 or 3 min with 5% ethanol reduces the activity by 45%).

In order to facilitate experimentation with rhodopsin kinase, we sought conditions under which it could be conveniently frozen, incubated, and assayed with retention of activity. Kinase samples lost 80% activity when frozen at liquid

[2] Several different chromatographic separations were tried in our attempts to purify rhodopsin kinase from the KCl extract, in addition to the successful approach adopted. Chromatography on CM-cellulose (Sigma) using 75 mM Tris-HCl, 1 mM $Mg(OAc)_2$, 1 mM DTT (pH 7.8) yielded a recovery of only 20% of the loaded activity, and this activity did not bind to the column. Changing the pH to 6.8 resulted in a complete loss in recoverable activity. Similarly, chromatography on phosphocellulose (Sigma) in this buffer at pH 7.5 yielded no recoverable activity.

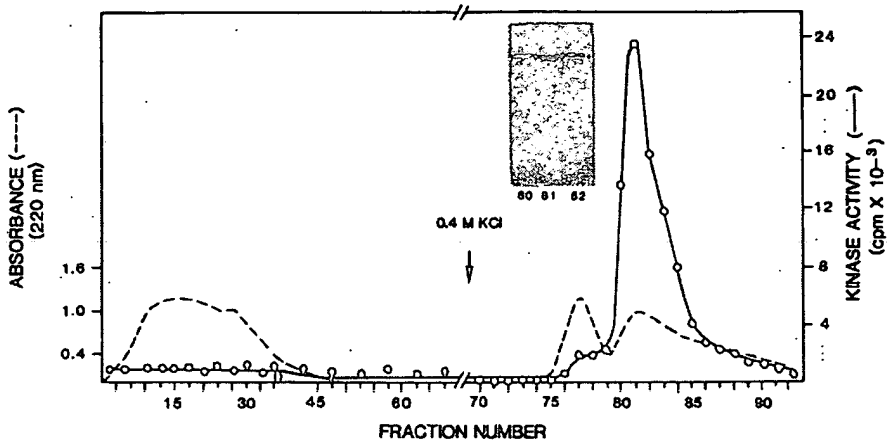

FIG. 3. **Hydroxyapatite chromatography of rhodopsin kinase.** The combined fractions containing rhodopsin kinase eluted from DEAE-cellulose (Fig. 2) were loaded on a hydroxyapatite column (1.0 × 10 cm) equilibrated with 75 mM Tris-HCl buffer (pH 7.8) containing 1 mM dithioerythritol and 1 mM $Mg(OAc)_2$. Then the column was washed with the same buffer, until the optical density at 220 of the effluent dropped below 0.05. The rhodopsin kinase fractions were then eluted with the same buffer containing 0.4 M KCl. 2-ml fractions were collected. All samples of kinase were desalted using reverse-phase chromatography as described under "Materials and Methods," before SDS-PAGE was performed using the Laemmli system (31) with 12% acrylamide gels. The *inset* shows stained gels of aliquots from fractions 80, 81, and 82 from the hydroxyapatite column which contain rhodopsin kinase activity.

nitrogen temperatures in water or inositol, and 30% in sucrose. No loss of activity was observed when kinase was frozen in adonitol. Similar losses of activity occurred when samples were frozen at −20 °C, except when adonitol was present, in which case no loss of activity occurred. The enzyme is stable for 2 h at 4 °C in adonitol and other solutions. Neither adonitol nor sucrose protect from activity loss at 30 °C, since 50% activity is lost after 2 h in water or the sugars. Solid sucrose and adonitol were added to kinase solutions which were then frozen at −20 °C and assayed at monthly intervals. In sucrose the enzyme lost an initial 30% activity and was stable to further storage. In adonitol there was no loss of activity over a period of months. We now routinely store our kinase frozen at −20 °C in 20% adonitol.

Effect of Cyclic Nucleotides and Inositol Phosphates on Rhodopsin Kinase Activity—Several compounds which change concentrations upon illumination of rod outer segments were tested for their effect on rhodopsin kinase activity (Fig. 4). cGMP affects rhodopsin kinase activity by less than 10% in the micromolar to millimolar concentration range. Similarly, cAMP and inositol 1,4,5-trisphosphate have no effect at concentrations below 0.1 mM. At 1 mM, these two compounds inhibited the kinase activity by about 20–30%. In contrast, D-*myo*-inositol-1-phosphate appears to stimulate rhodopsin kinase activity by about 20% at concentrations between 0.3 and 100 μM while it inhibits somewhat at higher concentrations.

Effects of Adenosine Analogues on Rhodopsin Kinase Activity—By using purified rhodopsin kinase, the K_m for rhodopsin was found to be 4 μM (Table II), a value equivalent to that reported earlier for a crude kinase preparation (13). Since Mg^{2+} has been found to affect kinase activity (13), the K_m and V_{max} for ATP were measured at two Mg^{2+} concentrations (Table II). As observed previously, the effect of high concentrations of Mg^{2+} is to inhibit the kinase, in the present case by increasing the K_m as well as by slightly reducing the V_{max}.

A variety of analogues of ATP were tested for their effect on the kinase activity (Table II). Adenosine, for example, competes with ATP with a K_i of 4 μM, a value equivalent to that of the K_m of ATP (Fig. 5). Similarly, AMP and ADP

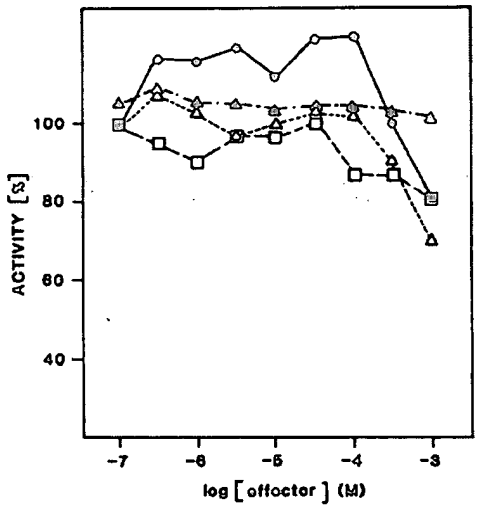

FIG. 4. **Influence of cyclic nucleotides and inositol derivatives on rhodopsin kinase activity.** The activity of rhodopsin kinase is plotted as a function of the concentration of the effector where 100% is the activity of rhodopsin kinase with no added effectors. ⊙, D-*myo*-inositol-1-monophosphate; □, D-*myo*-inositol-1,4,5-trisphosphate; △, cAMP; ▲, cGMP.

compete with almost equivalent K_i values. Substitution of sulfate for the phosphate of AMP reduces the inhibitory effect, but it remains a good inhibitor. Other substitutions in the 5′ position produce only poor inhibitors. Removing the hydroxyl in the 2′ position of the ribose moiety (2′-deoxyadenosine) abolishes its inhibitory effects, while removing the 3′-hydroxyl-(3′-deoxyadenosine) has little effect. Removing both hydroxyl groups (2′,3′-dideoxyadenosine) yields a good inhibitor although the K_i is increased somewhat. Other modifications tested yield only poor inhibitors. The analogues tested with modifications in the purine moiety of adenosine were also poor inhibitors of rhodopsin kinase activity. Dinucleotides have no effect on the kinase activity.

TABLE II

Effect of changes in the adenosine moiety on the K_m and V_{max} of substrates and inhibitors of rhodopsin kinase

Substrate	K_m	V_{max}	V_{max}/K_m*
	μM	*nmol/min/mg*	$s^{-1}\ M^{-1} \times 10^{-6}$
Rhodopsin (washed by 5 M urea)	4 ± 1.1	700	0.2
ATP at 1 mM Mg^{2+}	1.6 ± 0.7	638	0.46
ATP at 10 mM Mg^{2+}	5.2 ± 0.5	608	0.14
GTP at 2 mM Mg^{2+}	1000 ± 120	10	0.00001
Inhibitors			K_i
			μM
Adenosine			4 ± 0.4
Analogues, ribose 5′ position			
AMP			5 ± 1.1
ADP			12 ± 0.7
5′-Deoxyadenosine			10 ± 0.2
S-Adenosyl-L-methionine			>1000
CoA			>5000
Methylene adenosine 5′-triphosphate			>1000
Imidoadenosine 5′-triphosphate			>1000
Adenosine 5′-monosulfate			80 ± 2.1
2′ or 3′ position			
2′-Deoxyadenosine			No effect
3′-Deoxyadenosine			16 ± 2.1
Adenosine 2′-deoxy-3:5-monophosphate			No effect
Adenosine 2′:3′-monosphosphate			No effect
Adenosine 3′:5′-monophosphate			>3000
Adenosine 9-arabinofuranoside 5′-monophosphate			>3000
2′,3′-Dideoxyadenosine			65
Adenosine 5′-monophosphate (periodate oxidized/borohydride reduced)			>1000
Analogues, purine			
GMP			>5000
Guanosine			No effect
GDP			No effect
Xantosine 5′-monophosphate, hypoxanthine 9-arabinofuranoside			No effect
Inosine monophosphate			No effect
1-N^6-Ethenoadenosine monophosphate			No effect
Adenosine N^1-oxide			>5000
Dinucleotides			
NAD, NADH			No effect
NADP, NADPH			No effect

* V_{max}/K_m were calculated assuming M_r of rhodopsin kinase equal 70,000.

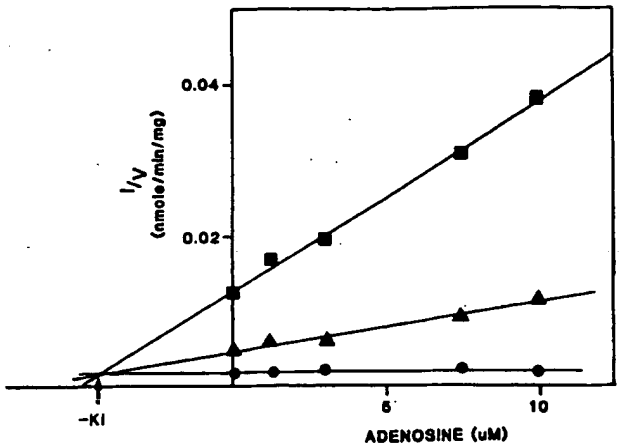

FIG. 5. **The effect of adenosine on rhodopsin kinase activity.** Rhodopsin kinase was assayed as described under "Materials and Methods" with different concentrations of adenosine (as indicated in figure). Concentrations of [γ-^{32}P]ATP used were 16.7 μM (●), 3.3 μM (▲), and 1 μM (■).

DISCUSSION

Molecular Properties of Rhodopsin Kinase—Rhodopsin kinase behaves as a soluble protein which can be extracted by either low or high ionic strength buffers. Elution of rhodopsin kinase from a C_4 HPLC column required 50% isopropanol, whereas bovine serum albumin, a protein with a similar molecular weight, requires only 30% isopropanol. This is consistent with the amino acid analysis of the purified rhodopsin kinase preparation (data not shown) and this suggests that rhodopsin kinase is more hydrophobic than bovine serum albumin.

We find the molecular weight of purified rhodopsin kinase to be M_r = 70,000 by SDS-PAGE. While several studies have included molecular weight determination of rhodopsin kinase by gel filtration (10, 11, 35, 37), these results have ranged from 52,000 (10) to 79,000 (11). We reexamined the molecular weight by gel filtration using a system in which the kinase activity elutes in the middle of the separation range of the system. We find a molecular weight of 67,000 which is consistent with the 69,000 observed by Kühn (35) and 68,000 observed by Lee *et al.* (37). Since the molecular weights observed by SDS-PAGE and gel filtration are the same, it is clear that no subunit structure is needed for rhodopsin kinase activity. We observed only a minor peak eluting around 50,000. This may represent the kinase activity reported by Shichi and Somers (10) and for which there is also some evidence in the gel filtration profile of Lee *et al.* (37). No significant quantities of this component are found when inhibitors of proteolysis are employed. We suspect that this M_r = 50,000 protein, which shows rhodopsin kinase activity, is a proteolytically modified form of rhodopsin kinase.

Effect of Cyclic Nucleotides and Inositol Phosphates on Rhodopsin Kinase Activity—Results from previous studies of the effect of cyclic nucleotides have been variable. Swarup and Garbers (19) found stimulation of phosphorylation of rhodopsin by cGMP at >5% bleach while at 1% bleach, a small inhibition of rhodopsin phosphorylation was observed. Shuster and Farber (20) observed a marked decrease in phosphorylation of rhodopsin in the presence of cGMP, but cAMP had almost no effect. A variety of other compounds have been examined for their effect on the phosphorylation of rhodopsin. However, since most of these studies utilized either whole ROS or relatively crude extracts containing rhodopsin kinase activity, we have reinvestigated the effects of these compounds using our assay system employing purified rhodopsin kinase and urea-washed ROS membranes. The marked effect of light on the metabolism of polyphosphoinositides in ROS (38) prompted us to examine the effect of two phosphoinositols as well. In this system, there are no other soluble enzymes that can interfere with either the kinase, the rhodopsin, or the components being tested for their effect on rhodopsin kinase. We found only a small effect of any of the compounds tested on rhodopsin kinase activity. We conclude that these compounds, *i.e.*, cAMP, cGMP, D-*myo*-inositol-1-monophosphate and D-*myo*-1,4,5-trisphosphate have no direct role in regulating rhodopsin kinase activity in the outer segment; however, they could have an indirect role by regulating other systems in the outer segments.

Effects of ATP Analogues on Rhodopsin Kinase—In this study, naturally occurring ATP analogues were employed to study rhodopsin kinase in order to determine the specificity of the ATP binding site of the rhodopsin kinase, and in order to evaluate what features of the nucleotide would be important

for future studies with covalent inhibitors. We also wanted to compare the effects of these analogues on rhodopsin kinase to their effects on other protein kinases. We have identified analogues which can block kinase activity in whole ROS as well as in a highly purified reconstituted system. The kinetic parameters are essentially the same for the purified kinase as for the kinase extract used previously (13) in which only about 10% of the protein in the extract was rhodopsin kinase. This indicates that results obtained earlier are valid and were not affected by interference from other components in the extract. Similar results were observed for the M_r = 52,000 kinase isolated by Schichi and Somers (10).

Protein kinases vary in their preference for high energy phosphate substrates. Some use ATP much more effectively than GTP (cAMP- and cGMP-dependent protein kinases, protein kinase C, hormone-stimulated ribosomal protein S6 kinase) (39–43). Others prefer GTP to ATP (growth-associated histone H1 kinase, K_m = 58 μM for ATP and 1.4 μM for GTP) (44). The K_m values for the high energy phosphate vary from 3.1 μM for protein kinase A up to 380 μM for phosphorylase kinase (45, 46). Rhodopsin kinase strongly prefers ATP as a substrate yielding a turnover for ATP about 40,000 times that of GTP (as determined from the ratio of their respective K_m values). This high affinity for ATP is based on the interaction between the nucleotide and the enzyme rather than the polyphosphate binding to the enzyme. This is indicated by the strong preference for ATP as compared to GTP as well as by the ability of adenosine to inhibit the kinase competitively with a K_i similar to the K_m of the enzyme.

An earlier study showed that rhodopsin kinase is inhibited by adenosine (10). We reinvestigated the effect of adenosine on rhodopsin kinase in order to determine the nature of this inhibition. The data presented in Fig. 5 demonstrate that adenosine is a pure competitive inhibitor with respect to ATP with a K_i of 4 μM similar to that of the K_m for the kinase for ATP. Other analogues of ATP were examined for their effect on rhodopsin kinase in order to give us information about the ATP binding pocket of rhodopsin kinase. As shown in Table II, modification of the 5′ position of adenosine by phosphorylation, sulfonation, or removal of the hydroxyl group has little effect on the K_i (*e.g.* K_i for AMP is 5 μM); see "Results" for AMP, ADP, adenosine 5′-monosulfate, 5′-deoxyadenosine). However, substitutions like *S*-adenosyl-L-methionine, dinucleotide (NAD^+ or NADH) or CoA dramatically change the K_i for rhodopsin kinase probably due to steric interactions. These data are quite different from those reported for cAMP- and cGMP-dependent protein kinase where changing the charge in the 5′ position (via phosphorylation) has a major effect on the interaction between enzyme and nucleotide (47). For this reason, the K_i values for adenosine for cAMP- and cGMP-dependent protein kinase are 0.54 and 1.7 mM, respectively, much higher than the K_m for ATP. On the other hand, the K_i of adenosine for calmodulin-dependent protein kinase is lower (250 μM (48)). For rhodopsin kinase, the charge does not play a big role, although some modifications in this region disrupt the binding to the kinase. For example, the nonhydrolyzable analogs AMPPNP and AMPPCP have lost most of their inhibitory effect for rhodopsin kinase while for other kinases they are effective inhibitors.

For cAMP-dependent protein kinase, cGMP-dependent protein kinase, and calmodulin-dependent protein kinase, the 3′-deoxy derivative of ATP has an even higher affinity for the kinase than ATP while the 2′-deoxy derivative has a lower affinity (47, 48). For rhodopsin kinase, these same modifications yield similar but not identical effects. The 3′-deoxy derivative binds almost as well as ATP while the 2′-deoxy derivative has completely lost its ability to bind. The 2′,3′-dideoxy derivative is a fairly good inhibitor whereas substituting a ribose isomer, adenosine 9-arabinofuranoside-5′-monophosphate again destroys the ability to inhibit. Another analogue is missing the 2′-3′ carbon-carbon bond, but retains the hydroxyl groups (adenosine 5′-monophosphate (periodate oxidized borohydride reduced)) and this compound also has little inhibitory effect. From these data, it is not clear whether the critical component for binding to the kinase is the hydroxyl group at position 2′ or the conformation of the ribose moiety or some combination of these two. It is possible that there is a carboxylic residue around the 2′ position in the active site of rhodopsin kinase similar to enzymes which have a Rossmann domain (49) because the presence of phosphate causes a loss in affinity for the enzyme (*e.g.*, 3′,5′-cAMP *versus* 2′,3′-cAMP).

Examining another region of the ATP molecule, we tested a few analogues with changes in the purine moiety. None of these analogues was an effective inhibitor of rhodopsin kinase, suggesting that the adenine moiety itself is very specifically recognized by rhodopsin kinase and not only by simply the appropriate conformation around the *N*-glycosylated bond. To summarize, it appears that rhodopsin kinase's ATP domain has similarities to those of cAMP- and cGMP-dependent protein kinase and calmodulin-dependent protein kinase, although it appears that the ribose moiety must be in the proper conformation for binding. It is also likely that the triphosphate chain has some special conformation acceptable in the active site of rhodopsin kinase since some substitutions yield poor inhibitors. The ATP-binding requirements of rhodopsin kinase therefore appear to be more specific than that of other kinases.

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EXHIBIT B

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Purification of a Novel Phospholipase A_2 from Bovine Seminal Plasma*

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Phospholipases A_2 are enzymes believed to play important roles in numerous physiological systems including sperm cell maturation. Relatively little work has, however, been devoted to study these enzymes in seminal plasma. We therefore undertook the purification and characterization of this enzyme from bovine seminal plasma. After a 330-fold purification, an activity corresponding to a protein of 100 kDa was identified by gel filtration. SDS-polyacrylamide gel electrophoresis analysis of the purified fraction revealed the presence of a 60-kDa band that comigrated with the activity during ion-exchange and gel filtration chromatography as well as polyacrylamide gel electrophoresis. The enzyme possessed a pH optimum around pH 6.5 and was calcium-dependent. Using isoelectric focusing, its isoelectric point was determined to be 5.6 ± 0.07. The enzymatic activity was resistant to *p*-bromophenacyl bromide, but was sensitive to gossypol and dithiothreitol. The enzyme was 2 orders of magnitude more active toward micelles formed with deoxycholate than with Triton X-100. Slight differences in the specificity toward head groups and/or *sn*-2-side chains were found in both assay systems. The enzyme was acid-labile and did not display affinity for heparin. It would therefore appear that the phospholipase A_2 form isolated from bovine seminal plasma is of a novel type.

Phospholipases A_2 (PLA_2)[1] are ubiquitous enzymes capable of hydrolyzing the *sn*-2-position of phospholipids. Most PLA_2 characterized to date belong to either one of two main groups: high and low molecular mass PLA_2 (1, 2). High molecular mass PLA_2, also called cytoplasmic PLA_2 ($cPLA_2$), are 85-kDa proteins found in the cytoplasm of several cell types (3–6). They are specific for arachidonic acid (6) and possess limited lysophospholipase (7, 8) and phospholipase A_1 (9) activities. Low molecular mass PLA_2 ($sPLA_2$) form a family of homologous enzymes with molecular masses ranging from 14 to 20 kDa that are found in several secretory fluids as well as in the cytoplasm of various cell types (1, 2, 10). PLA_2 are believed to be important regulatory enzymes in numerous physiological systems such as inflammation, membrane remodeling, and cell signalization (11). Several PLA_2 that do not belong to either category have also been identified in various tissues and organisms (12–19).

In the reproductive system, PLA_2 are widely accepted to play a major role in the late maturational events of spermatozoa, particularly in the acrosomal reaction (20–23). The acrosomal reaction is a multifusion process that permits the release of hydrolytic enzymes, which are required for spermatozoa to penetrate the acellular layers surrounding the oocyte (24).

Although several studies have been undertaken to characterize the PLA_2 present in the spermatozoa and seminal plasma of various species (25–30), only the enzyme from human seminal plasma has been purified to homogeneity and sequenced (31) so as to conclusively assign it to a particular PLA_2 group. The enzyme was found to be a 14-kDa protein, identical to the synovial enzyme (32), suggesting the same might be true of other mammalian species.

In bovine seminal secretions, the enzyme was partially purified, but was not characterized enough to assign it to a particular PLA_2 group (30). To determine the exact type(s) of PLA_2 present in bovine seminal plasma and to assess the generality of the occurrence of $sPLA_2$ in mammalian seminal plasma, we purified and characterized the major PLA_2 activity from bovine seminal plasma.

EXPERIMENTAL PROCEDURES

Materials

Sephacryl S-300, butyl-Sepharose Fast Flow, and Q-Sepharose Fast Flow were purchased from Pharmacia Biotech (Baie d'Urfée, Québec, Canada). Electrophoresis reagents (including ampholytes) were obtained from Bio-Rad. Heparin, gossypol, and *p*-bromophenacyl bromide were from Sigma. Phosphatidylcholine (PC) (L-α-1-palmitoyl-2-[^{14}C]linoleoyl (specific activity of 55.6 mCi/mmol) and L-α-1-palmitoyl-2-[^{14}C]arachidonyl (specific activity of 52.6 mCi/mmol)) and phosphatidylethanolamine (PE) (L-α-1-palmitoyl-2-[^{14}C]arachidonyl (specific activity of 55.6 mCi/mmol)) were obtained from New England Nuclear (Mississauga, Ontario, Canada). The scintillation fluid (Universol) was purchased from ICN (Montreal). Aluminum-backed silica gel TLC plates were from Whatman (Maidstone, United Kingdom). Recombinant PLA_2 (porcine pancreatic and *Crotalus atrox*) were from Sigma. Dialysis membranes were from Spectrum Medical Industries, Inc. (Houston, TX). Ultrafiltration membranes were from Amicon, Inc. (Beverly, MA). All other chemicals used were of analytical grade and were purchased from commercial suppliers. Bovine semen was a generous gift from the Centre d'Insémination Artificiel du Québec (St.-Hyacinthe, Québec, Canada).

Phospholipase A_2 Assay

Enzymatic activity was assayed using *sn*-2-radiolabeled 2-arachidonyl-PE unless specified otherwise. The substrate (20,000 cpm/tube, 1.7 μM) was evaporated under nitrogen and resuspended in buffer A (50 mM

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[1] The abbreviations used are: PLA_2, phospholipase(s) A_2; $cPLA_2$ cytoplasmic phospholipase(s) A_2; $sPLA_2$, low molecular mass phospholipase(s) A_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; pBPB, *p*-bromophenacyl bromide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

Tris-HCl, 0.02% NaN_3, pH 7.4) containing 10 mM sodium deoxycholate. The substrate solution was vortexed and mixed for 20 min. Ten μl of substrate solution was added to each assay tube. A typical reaction mixture (final volume of 100 μl) consisted of 1 mM $CaCl_2$ and 1 mM sodium deoxycholate in buffer A. After 30 min at 37 °C, the reaction was stopped by adding 200 μl of chloroform/methanol (2:1) containing 2 μg/ml fatty acid tracer and 50 μl of 4 M KCl. The assay tubes were then centrifuged, and the lower phase was applied onto a silica TLC plate, which was then developed in petroleum ether/ether/acetic acid (85:15:1). The fatty acids were visualized with iodine, and the stained spots were cut into scintillation vials. The scintillation fluid was then added, and the radioactivity was determined in a liquid scintillation counter.

Purification Methods

Seminal Plasma Preparation—Pools of bovine ejaculates were centrifuged at low speed (300 × *g*) to remove spermatozoa. The supernatant was then preserved at −20 °C and used for purification within 2 weeks.

Butyl-Sepharose Chromatography—Ten ml of frozen seminal plasma was thawed, adjusted to 0.1 M choline chloride, and centrifuged at 10,000 × *g*, and the supernatant was loaded (2 ml/min) on a 2.5 × 10-cm butyl-Sepharose column equilibrated in buffer A containing 0.1 M choline chloride. The column was then washed at 7 ml/min with 700 ml of equilibration buffer followed by 350 ml of 5 M urea in buffer A (Fraction I).

Sephacryl S-300 Chromatography—Fraction I was concentrated by ultrafiltration (pore size of 10,000; Amicon, Inc.) and applied to a 1.5 × 110-cm Sephacryl S-300 column (4 °C) equilibrated in buffer A containing 0.15 M choline and 0.15 M NaCl. Fractions (5.8 ml) were collected at a flow rate of 0.3 ml/min. The fractions under the activity peak were pooled and concentrated (Fraction II). Calibration of the column was performed under the same conditions by passing RNase A, ovalbumin, and bovine serum albumin.

Q-Sepharose Chromatography—Fraction II was applied to a Q-Sepharose column (1 × 1 cm) coupled to a fast protein liquid chromatography system and equilibrated in buffer A (without NaN_3) containing 0.2 M NaCl. The active fractions were eluted with a 0.2–1 M NaCl gradient in buffer A. Fractions (1 ml) were collected at a flow rate of 1 ml/min.

Characterization

A partially purified (190-fold) fraction, obtained by an alternative lower yield approach, was preserved at −20 °C in 25% glycerol and used for all characterization studies unless otherwise specified. The substrate used was arachidonyl-PE unless specified otherwise.

pH Dependence—The following buffers were used for pH dependence studies: pH 4–5, 50 mM sodium acetate; pH 6.5–7, 50 mM MES; pH 7.5–8.5, 50 mM Tris-HCl; pH 9–10.5, 50 mM ethanolamine; and pH 11–11.5, 50 mM CAPS. The reaction was carried out at 22 °C.

Isoelectric Focusing—Isoelectric focusing was performed at 22 °C for 7000 V-h on a post Sephacryl S-300 aliquot adjusted to 5 M urea and 2% ampholytes. The gel rods (0.3 × 13 cm) consisted of 4% acrylamide, 2% ampholytes, pH 3–10, 2% Triton X-100, and 5 M urea. After completion of the electrophoresis, the gel rod was cut into 24 pieces, and proteins were eluted in 500 μl of H_2O/piece at 4 °C for 16 h on an orbital shaker.

Inhibition Studies—For inhibition studies, PLA_2-containing fractions were preincubated with the indicated concentrations of inhibitor dissolved in dimethyl sulfoxide (pBPB and gossypol) or H_2O (dithiothreitol (DTT)) for 3 h (pBPB) or for 30 min (gossypol or DTT) at 37 °C in buffer A. The sample was diluted 10 times prior to the enzymatic assay so that the final concentration of dimethyl sulfoxide in the assay tube was 1%.

Protein Estimation—During purification, protein concentration in each fraction was estimated by monitoring the absorbance at 280 nm. Protein content in pooled fractions was determined according to Bradford (33).

SDS-PAGE and Related Techniques—SDS-polyacrylamide gel electrophoresis (PAGE) was performed essentially as described by Laemmli (34). PAGE was performed on a 6% gel according to Kramer *et al.* (35). The apparent molecular mass of the various protein bands was determined with the low molecular mass calibration kit from Pharmacia Biotech. Proteins were visualized using Coomassie Brilliant Blue R-250 (36).

RESULTS

Purification of Bovine Seminal PLA_2

Seminal plasma was first passed through a butyl-Sepharose resin (Fig. 1*a*). Extensive washing (14 column volumes) was required to remove all the weakly adsorbed proteins. The urea-

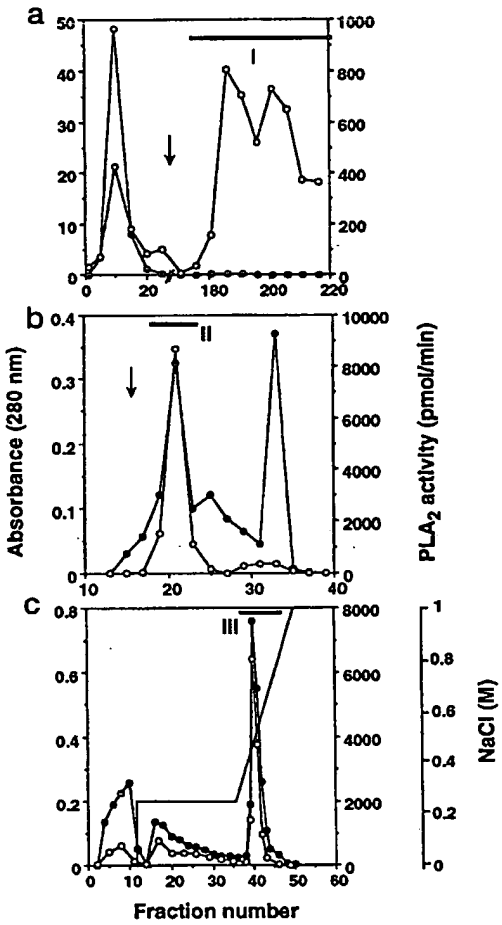

FIG. 1. **Purification scheme.** *a*, butyl-Sepharose chromatographic pattern; *b*, Sephacryl S-300 chromatographic pattern; *c*, Q-Sepharose chromatographic pattern. Chromatography was performed as described under "Experimental Procedures." The fractions under the *bar* were pooled and assayed for purity as indicated in Table I. Where appropriate, the gradients used are indicated. The *arrow* in *a* indicates the point of addition of the 5 M urea buffer. In *b*, the approximate void volume of the Sephacryl S-300 column is indicated by the *arrow*. ●, absorbance; ○, PLA_2 activity.

TABLE I
Purification summary

Aliquots of the various fractions obtained during purification were assayed for activity and protein content as described under "Experimental Procedures." One activity unit corresponds to 1 pmol of PE hydrolyzed per min.

Step	Activity	Protein	Specific activity	Yield	Purification
	units	*mg*	*units/mg*	%	*-fold*
Seminal plasma	24,000	730	33	100	1
Butyl-Sepharose FF (I)	28,000	6.6	4,200	120	130
Sephacryl S-300 (II)	32,000	3.9	8,200	130	250
Q-Sepharose FF (III)	11,000	1.0	11,000	45	330

FIG. 2. **SDS-PAGE of active pools obtained during purification.** The pooled fractions (~5 μg of protein/pool) were subjected to SDS-PAGE. *Lane 1*, 3 μg of molecular mass markers (LMW, Pharmacia Biotech); *lane 2*, seminal plasma; *lane 3*, Fraction I; *lane 4*, Fraction II; *lane 5*, Fraction III. The samples were adjusted with reducing SDS-PAGE buffer, boiled for 10 min, and loaded onto a 12% SDS-polyacrylamide Mini-Gel (Bio-Rad), which was then stained with Coomassie Brilliant Blue. The migration of the molecular mass markers corresponds (from top to bottom) to 94, 67, 43, 30, 20.1, and 14.4 kDa, respectively.

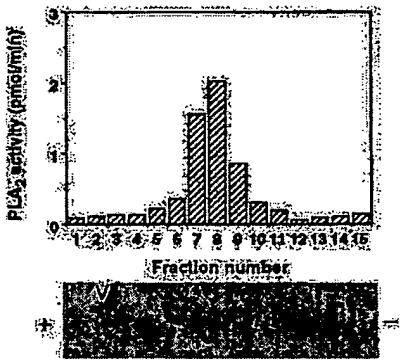

FIG. 3. **Native PAGE of purified PLA_2.** Ten μg of purified enzyme was loaded in each of two lanes of a polyacrylamide gel. After electrophoresis (100 min, 200 V), one lane was cut into 15 pieces, which were then eluted for 20 h (4 °C) in 500 μl of 0.1 M NH_4HCO_3, while the other lane was stained with Coomassie Brilliant Blue. The eluate of each fraction was assayed for PLA_2 activity. The *V* indicates the migration position of bovine serum albumin under the same conditions.

desorbed fractions (Fraction I) contained most of the recovered activity. Fraction I was concentrated and loaded onto a Sephacryl S-300 gel sieving column (Fig. 1*b*). A single active peak was obtained whose elution position corresponded to the behavior of a 100-kDa protein as determined by calibration of the column. The active peak was then concentrated and applied onto a Q-Sepharose ion exchanger. The activity was again eluted in one major activity peak, which well overlapped the protein pattern (Fraction III).

The purification results are summarized in Table I. This scheme resulted in a purification of 330-fold with a 45% recovery of the activity.

Characterization of Bovine Seminal PLA_2

The Purified Enzyme Behaves as a 60-kDa Protein on SDS-PAGE—The active fractions from the successive purification steps were analyzed by SDS-PAGE (Fig. 2) under reducing conditions. After a single purification step (Fraction I; *lane 3*), a main component at 60 kDa is visible. This component then persists throughout until the end of the purification procedure, where it is the only major band detectable by Coomassie Blue staining (Fraction III; *lane 5*).

The 60-kDa Band Is Responsible for the Activity—Fraction III was subjected to PAGE. Measurement of the activity eluted from the gel slices revealed that it was recovered at a position corresponding to the protein (Fig. 3).

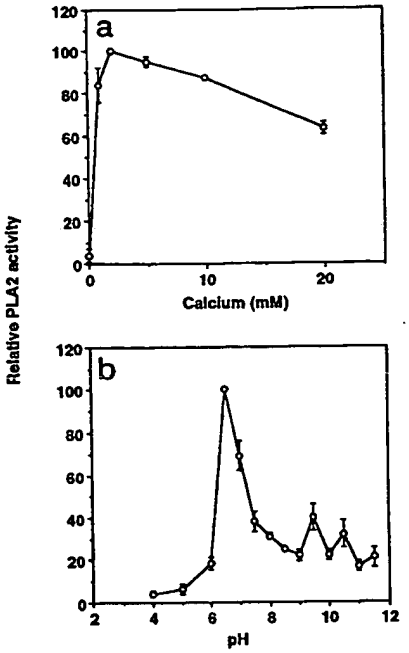

FIG. 4. **Calcium requirement and pH optima.** The dependence of seminal PLA_2 on calcium concentrations (as $CaCl_2$) (*a*) and pH (*b*) in the reaction media was determined as described under "Experimental Procedures." To generate 0 mM Ca^{2+}, 100 μM EGTA was added to calcium-free buffer A. Results are expressed as a percentage of the untreated enzyme and represent the means ± S.E. of three independent experiments.

Calcium Requirement and pH Optimum—In a manner similar to most phospholipases characterized thus far, the enzyme was calcium-dependent and was maximally active at ~2 mM calcium (Fig. 4*a*), while analysis of the pH dependence of the activity revealed a single activity maximum at pH 6.5 (Fig. 4*b*).

Sensitivity of Bovine Seminal PLA_2 to Known PLA_2 Inhibitors—Purified PLA_2 was resistant to pBPB, whereas the two positive controls, porcine pancreatic and *C. atrox* PLA_2, were inhibited (Fig. 5*a*). Seminal PLA_2 was inhibited by gossypol at inhibitor concentrations higher than those required to inhibit crotal PLA_2, but similar to those required to inhibit the porcine pancreatic enzyme (Fig. 5*b*). The porcine enzyme and seminal PLA_2 also shared similar sensitivities to the thiol reagent DTT (Fig. 5*c*); the sensitivity of the crotal enzyme toward DTT was not investigated in this study.

Determination of the Enzyme pI—To determine the pI of PLA_2, isoelectric focusing of a partially purified enzyme was performed (Fig. 6). The gel rod was cut into 24 pieces, which were then left to elute in H_2O. The supernatants were assayed for PLA_2 activity, and their pH was measured. Several (n = 8) such experiments revealed a single activity peak at pH 5.6 ± 0.07 (mean ± S.E.). Typical activity recoveries on the order of 10–20% were obtained. The true recovery is expected to be higher since the Triton X-100 concentration in the supernatants (~0.001% final concentration) inhibited the activity of a partially purified fraction by ~50% (data not shown).

Substrate Specificity of Seminal PLA_2—The substrate specificity was studied in the presence of phospholipid micelles consisting of either PC or PE and deoxycholate or Triton X-100. As summarized in Table II, PLA_2 was 2 orders of magnitude more active toward the deoxycholate-containing substrate than toward the Triton X-100-containing substrate or vesicular substrate (data not shown). In the presence of deoxycholate, the enzyme discriminated between the *sn*-2-fatty acid as it was less active toward PC carrying linoleoyl (1111 ± 98) than arachi-

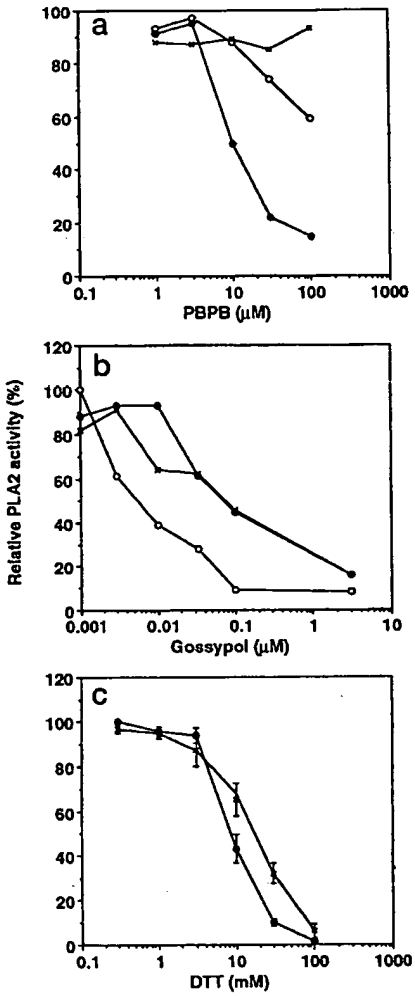

FIG. 5. **Sensitivity of the seminal enzyme to PLA_2 inhibitors.** Seminal (×), crotal (○), and porcine pancreatic (●) PLA_2 were pretreated with the indicated PLA_2 inhibitors as described under "Experimental Procedures." The PLA_2 activity of an aliquot (10-fold diluted) that underwent the appropriate treatment was then assayed over a 30-min period. The data shown here represent the mean of three independent determinations. For clarity, the standard error is shown only in *c*; for *a* and *b*, it was typically below 10% and never above 15% of the corresponding mean.

donyl (1716 ± 73). For a given *sn*-2-side chain, no selectivity was observed between PE/deoxycholate- or PC/deoxycholate-containing micelles as both substrates were hydrolyzed at similar rates, suggesting that the enzyme shows little, if any, head group specificity in this assay system. When micelles comprising Triton X-100 were used, however, head group specificities were observed. The ethanolamine phospholipid was cleaved more efficiently than the corresponding choline phospholipid (56 ± 5.8 *versus* 17 ± 1.5), although the total amount hydrolyzed remained much lower than when deoxycholate was present. Interestingly, the side chain specificity observed with deoxycholate-containing micelles was reversed when Triton X-100 micelles were used, as linoleyl was then preferred over arachidonyl (48 ± 1.7 *versus* 17 ± 1.5).

DISCUSSION

The seminal PLA_2 activity bound specifically to the butyl-Sepharose resin, thus permitting a 130-fold purification in a single step. Choline had to be included throughout this step to prevent the heparin-binding proteins, the main component of bovine seminal plasma (37), from strongly binding to the resin.

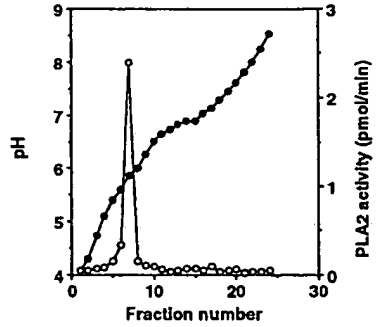

FIG. 6. **Isoelectric focusing of seminal PLA_2.** A partially purified aliquot was adjusted to 5 M urea and 2% ampholytes, applied onto an isoelectric focusing polyacrylamide gel rod, and subjected to isoelectric focusing. The gel rod was then cut into 24 equally sized pieces, which were incubated for 16 h in distilled water. The PLA_2 activity content of the supernatant was then determined. The pH in each fraction was measured using a glass electrode. ●, pH; ○, PLA_2 activity.

TABLE II
Substrate specificity

The partially purified enzyme was incubated with 3-palmitoylphospholipids (0.17 μM) bearing different ^{14}C-labeled *sn*-2-acyl groups and containing either ethanolamine or choline as head group. The substrate was prepared 20 min in advance and was diluted 10 times in the assay tube to yield the indicated detergent concentrations. The results represent the means ± S.E. of three independent experiments.

Phospholipid	Enzymatic activity	
	Deoxycholate (1 mM)	Triton X-100 (0.01%)
Arachidonyl-PE	2,000 ± 32	56 ± 5.8
Arachidonyl-PC	1,700 ± 73	17 ± 1.5
Linoleoyl-PC	1,100 ± 98	48 ± 1.7

Rechromatography of the unadsobed fraction did not permit further binding of the activity, thus suggesting the presence of another form of PLA_2, which was not further investigated in this study. Chromatography on both gel filtration and ion-exchange resins (Fig. 1, *b* and *c*) resulted in activity and protein absorbance patterns that eluted closely together, indicating that the major protein (absorbance at 280 nm) was also responsible for the activity. When analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue, a major 60-kDa band was visible in both chromatographic runs (Fig. 2). Further confirmation that the 60-kDa band was responsible for the activity was obtained by PAGE. Fractions that consisted of eluates of gel slices were assayed for PLA_2 activity, and again, the band intensity and the corresponding enzymatic activity variations matched closely (Fig. 3). Gel filtration revealed that the activity behaved as a 100-kDa protein (Fig. 1*b*), whereas SDS-PAGE analysis showed a 60-kDa band (Fig. 2). This discrepancy might be attributed to dimerization of the 60-kDa enzyme. This dimer appears stable since moderately stringent conditions (0.1% deoxycholate or 5 M urea) failed to shift the elution position of PLA_2 (data not shown). Since the omission of 2-mercaptoethanol did not change its behavior on SDS-PAGE (data not shown), it appears that the interaction is noncovalent. Consistent with the dimer hypothesis, the enzyme behaves on native PAGE as a much larger protein than bovine serum albumin despite a very similar pI (Fig. 3).

Binding to Q-Sepharose at pH 7.4 (Fig. 1*c*) as well as isoelectric focusing (Fig. 6) indicate that the enzyme is acidic. In comparison, most mammalian $sPLA_2$ are neutral to basic proteins, with one notable exception (10). $cPLA_2$, on the other hand, possess pI values similar to those of the seminal enzyme (Fig. 6) (8, 35). Besides this similarity, however, the seminal enzyme shares little in common with $cPLA_2$. Using two different assay systems, the seminal enzyme did not show the char-

acteristic specificity for arachidonylphospholipids found in high molecular mass PLA_2. In the Triton X-100 assay system, the seminal plasma PLA_2 activity toward *sn*-2-arachidonyl was ~3-fold lower than the activity toward linoleyl, whereas $cPLA_2$, in a similar assay system, displayed a 3-fold higher activity (5). Moreover, while $cPLA_2$ is inhibited by deoxycholate micelles relative to sonicated vesicles (4), the reverse is observed for the seminal enzyme (data not shown).

The resistance of the enzyme to pBPB supports the view that this enzyme is novel. pBPB inactivates $sPLA_2$ by alkylating a histidine residue located in the active site of the enzyme (38). It also inactivates $cPLA_2$ (39) by an unknown mechanism, which is likely to be quite different from $sPLA_2$ since $cPLA_2$ does not possess an active-site histidine (3). At the pBPB concentrations used, both enzyme types should be inactivated, and yet, the seminal enzyme is unaffected. As expected, the two PLA_2 controls, the type I porcine pancreatic and the type II *C. atrox* enzymes, were inactivated (Fig. 5*a*). The greater resilience of the crotal enzyme is most likely due to its tendency to shield its active site through dimerization (40, 41). This raises the possibility that seminal PLA_2 possesses a histidine or some other susceptible residue in its active site, which would be completely shielded from the environment in the absence of substrate and/or Ca^{2+}.

Despite this resistance, some common structural features between pancreatic and seminal PLA_2 are suggested by the inhibition patterns of DTT and gossypol. The pancreatic enzyme is inhibited by gossypol at concentrations very close to those required to inhibit the porcine enzyme (Fig. 5*b*). Although the precise structural modifications induced by gossypol are unknown, the similar concentrations required to inhibit pancreatic PLA_2 and the seminal enzyme suggest some common structural elements. This resemblance appears to be quite specific as the inhibition pattern of the crotal enzyme, which shares strong structural homologies with the pancreatic enzyme (1, 2), is completely different. The shared DTT sensitivities (Fig. 5*c*) further support the view that common features between mammalian $sPLA_2$ and seminal PLA_2 exist. Biochemical characterization revealed that seminal PLA_2 shows catalytic properties common to most $sPLA_2$ identified so far: the enzyme is Ca^{2+}-dependent (Fig. 4*a*) and is optimally active in the neutral to alkaline pH range (Fig. 4*b*) (42).

The substrate selectivity profile of purified PLA_2 is also reminiscent of mammalian $sPLA_2$ (43, 44). For instance, these enzymes are activated by the introduction of negative charges (as with deoxycholate *versus* Triton X-100) in the lipid substrate, most likely due to the accumulation of positive charges near the phospholipid-binding site (45). In the absence of deoxycholate, for a given acyl side chain, they are more active toward the anionic phospholipid PE than toward the zwitterionic phospholipid PC (43).

Beside these catalytic similarities, major structural differences appear to exist between these enzymes. For instance, mammalian $sPLA_2$ are low molecular mass (14–20 kDa) and mostly basic proteins, whereas the seminal enzyme possesses a 60-kDa mass and an acidic pI. Pancreatic PLA_2 and the human seminal/synovial enzyme demonstrate affinity for heparin (46–48), while bovine seminal PLA_2 does not (data not shown). Moreover, $sPLA_2$ are resistant to acidic conditions as relatively good recoveries are routinely obtained following chromatography performed under acidic conditions (49–51), whereas the major PLA_2 activity found in seminal plasma is acid-labile (data not shown).

The seminal enzyme displays a specific activity (under suboptimal conditions) of ~0.01 μmol/min/mg, which is rather low compared with that of low molecular mass PLA_2 (for instance, ~40 and 1500 μmol/min/mg for bovine pancreatic and *Naja naja* venom PLA_2, respectively) or with that of $cPLA_2$ (~0.6 μmol/min/mg) (4). The activity range of these well characterized PLA_2 thus covers 5 orders of magnitude. The resistance of seminal PLA_2 to pBPB (Fig. 5*a*) might indicate that it acts via a different, less efficient catalytic mechanism than the established enzymes. The lower catalytic efficiency of bovine seminal PLA_2 could be required for its proper function in seminal plasma. Alternatively, it could possess some yet undetermined advantages over other types of PLA_2 that would render it better suited to the particularity of the bovine reproductive physiology.

These results differ significantly from those reported previously concerning bovine (30) or human (28, 29, 31, 47, 52) seminal plasma PLA_2. The major human seminal plasma PLA_2 has been found to be identical to the synovial enzyme (31, 32). A minor form that was not recognized by the anti-synovial PLA_2 antibody was also reported (31). In the bovine species, the preliminary characterization of the enzyme published previously (30) did not permit any definitive conclusions to be drawn as to the nature of the seminal enzyme. Two different enzymatic activities were partially purified from seminal vesicle secretions. SDS-PAGE of the most purified fraction showed a doublet migrating as 14–16-kDa proteins. This enzyme may represent a minor PLA_2 form. The human prostate enzyme has also been partially purified and characterized (53). Overall, its biochemical properties appear to be quite distinct from those of bovine seminal plasma PLA_2.

The activities found in bovine, ram, and porcine seminal plasma amount to ~1, 10, and 0.03%, respectively, of the human seminal plasma PLA_2 activity (29), suggesting that qualitative differences might exist between the PLA_2 types found in these species. The structural characterization of the enzyme that is currently underway should reveal the reasons behind these differences.

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EXHIBIT C

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Isolation of a cDNA Coding for L-Galactono-γ-Lactone Dehydrogenase, an Enzyme involved in the Biosynthesis of Ascorbic Acid in Plants

PURIFICATION, CHARACTERIZATION, cDNA CLONING, AND EXPRESSION IN YEAST*

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L-Galactono-γ-lactone dehydrogenase (EC 1.3.2.3; GLDase), an enzyme that catalyzes the final step in the biosynthesis of L-ascorbic acid was purified 1693-fold from a mitochondrial extract of cauliflower (*Brassica oleracea*, var. botrytis) to apparent homogeneity with an overall yield of 1.1%. The purification procedure consisted of anion exchange, hydrophobic interaction, gel filtration, and fast protein liquid chromatography. The enzyme had a molecular mass of 56 kDa estimated by gel filtration chromatography and SDS-polyacrylamide gel electrophoresis and showed a pH optimum for activity between pH 8.0 and 8.5, with an apparent K_m of 3.3 mM for L-galactono-γ-lactone. Based on partial peptide sequence information, polymerase chain reaction fragments were isolated and used to screen a cauliflower cDNA library from which a cDNA encoding GLDase was isolated. The deduced mature GLDase contained 509 amino acid residues with a predicted molecular mass of 57,837 Da. Expression of the cDNA in yeast produced a biologically active protein displaying GLDase activity. Furthermore, we identified a substrate for the enzyme in cauliflower extract, which co-eluted with L-galactono-γ-lactone by high-performance liquid chromatography, suggesting that this compound is a naturally occurring precursor of L-ascorbic acid biosynthesis *in vivo*.

Vitamin C or ascorbic acid (L-AA)[1] is an important metabolite for most living organisms present in millimolar concentrations and is well known for its antioxidant properties. Its precise functions in plants is still poorly understood, although it is known to play an important role in the antioxidant system that protects plants from oxidative damage resulting from biotic and abiotic stresses as well as being a cofactor for a number of hydroxylase enzymes.

L-AA is synthesized by all higher plants and by nearly all higher animals except humans, other primates, guinea pigs, bats, and some birds (1–3). L-AA has also been reported to be present in a number of yeasts (4), but several reports suggest that L-AA analogues, rather than L-AA, are present in microorganisms (5–7).

The biosynthesis of L-AA follows different pathways in the animal and the plant kingdom. In animals, D-glucose serves as the first committed precursor in the biosynthesis of L-AA and the last step in the pathway is catalyzed by a microsomal L-gulono-γ-lactone oxidase (EC 1.1.3.8), which oxidizes L-gulono-γ-lactone (L-GuL) to L-AA. This enzyme has been isolated and characterized from rat, goat, and chicken (8, 9).

Despite the importance of L-AA in plants, the biosynthetic pathway has still not been established, although current evidence suggests the existence of two discrete routes. A biosynthetic pathway from D-galactose proceeding via L-galactono-γ-lactone (L-GL) has been proposed as long ago as 1954 by Isherwood *et al.* (10) and Mapson *et al.* (11), based on initial studies of the oxidation of L-GL to L-AA by the enzyme L-galactono-γ-lactone dehydrogenase (GLDase). GLDase activity has been described (11–13) in plants such as pea, cabbage, cauliflower florets, and potato, and recently Ôba *et al.* (14) reported a purification of this enzyme from sweet potato roots. Loewus (15) has proposed an alternative pathway in which L-AA is synthesized from D-glucose via L-sorbosone. The presence of an enzyme able to convert L-sorbosone to L-AA with concomitant reduction of NADP was demonstrated in bean and spinach leaves (16, 17). Conceivably, these distinct routes might be present in different subcellular compartments or in different plant species.

Here, we report the purification and characterization of GLDase from cauliflower florets, followed by isolation and sequencing of the corresponding cDNA. This is the first description of a gene coding for an enzyme involved in the biosynthesis of L-AA in plants. The GLDase cDNA has furthermore been expressed in an active form in yeast, and we have strong indications that the substrate for GLDase, L-GL is naturally present in plant extracts. These findings emphasize for the first time the physiological relevance of the biosynthetic pathway proposed by Isherwood *et al.* and Mapson *et al.* (10, 11).

EXPERIMENTAL PROCEDURES

Materials—Sephacryl SF-200, DEAE-Sepharose, and phenyl-Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden). L-Galactono-γ-lactone, D-galactono-γ-lactone, D-gulono-γ-lactone, L-gulono-γ-lactone, L-mannono-γ-lactone, D-galactonic acid, D-glucuronic acid,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z97060.

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[1] The abbreviations used are: L-AA, L-ascorbic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; L-GL, L-galactono-γ-lactone; L-GuL, L-gulono-γ-lactone; GLDase, L-galactono-γ-lactone dehydrogenase; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).

D-gluconic acid, and *p*-hydroxymercuribenzoic acid were from Sigma. D-Erythronic-γ-lactone, D-xylonic-γ-lactone, and *N*-ethylmaleimide were purchased from Aldrich. Restriction enzymes were from Pharmacia and [α-^{32}P]dCTP was from Amersham (Aylesbury, United Kingdom). Cauliflowers (*Brassica oleracea*, var. botrytis) were obtained from a field nearby Gent and kept at 4 °C until use.

Extraction—Cauliflower florets (7.5 kg) were cut into small pieces and homogenized in a pre-cooled blender in ice-cold buffer A (400 mM sucrose, 100 mM sodium phosphate buffer, pH 7.4) at 1 liter/kg fresh weight. The homogenate was passed through four layers of Miracloth tissue (Calbiochem-Novabiochem, La Jolla, CA), and centrifuged at 13,500 × *g* for 45 min in a GS3 rotor. The pellet containing the mitochondria (approximately 250 g of material) was stored at −70 °C until further use. The crude, frozen mitochondrial pellet was gently thawed in a microwave oven and resuspended in 1/10 volume (750 ml) of buffer A. Cold acetone (−20 °C) was slowly added while stirring (10 × volumes) and the mixture was allowed to stand for 30 min at 4 °C. Precipitated proteins were then collected by filtration through pre-filter paper (A15; Millipore, Bedford, MA) and resuspended in 1/10 volume of buffer B (40 mM Tris-HCl, pH 9.0) followed by 5 h dialysis against 10 volumes of buffer B. The denatured proteins were removed by centrifugation (10,000 × *g* for 15 min). GLDase was then purified from the supernatant and designated as the protein extract, using the protocol described below ("Enzyme Purification"). All manipulations concerning the preparation of extracts and enzyme purification were carried out at 4 °C, unless stated otherwise.

GLDase Assay—GLDase activity was measured spectrophotometrically by following the L-GL-dependent reduction of cytochrome *c* at 550 nm and 22 °C. The reaction mixture (1 ml) consisted of enzyme extract, cytochrome *c* (1.5 mg/ml), and L-GL (4.2 mM) in 0.05 M Tris-HCl buffer (pH 8.4). Under these conditions the reaction rate was linear with respect to time for an initial period of at least 15 min. One unit of enzyme activity was defined as the amount that oxidized 1 μmol of L-AA/min. This corresponds to the reduction of 2 μmol of cytochrome *c* as described by Ôba *et al.* (13). Substrate specificity assays were carried out as described above using 4.2 mM of the different substrates to be tested.

Enzyme Purification—The protein extract (from 250 g of mitochondrial pellet) was loaded onto a DEAE-Sepharose column (5 × 12 cm) equilibrated with buffer B. After washing with 4 column volumes of buffer B at 60 ml/h, elution was carried out with 0.5 M NaCl in the same buffer. Fractions of 8 ml were collected at a flow rate of 60 ml/h, and fractions containing GLDase activity were pooled and ammonium sulfate was added to a concentration of 1 M. The extract was then loaded on a phenyl-Sepharose CL-4B column (2.2 × 15.0 cm) equilibrated in buffer C (1 M ammonium sulfate, 25 mM sodium phosphate, pH 7.0). After washing with 2 column volumes of buffer C, elution was carried out at 30 ml/h by mixing buffer C with a 600-min linear gradient of 80% ethylene glycol in 25 mM sodium phosphate (pH 7.0).

Fractions containing GLDase activity were again pooled, concentrated to 10 ml by ultrafiltration using a PM-10 membrane (Amicon, Beverly, MA), and then applied onto a Sephacryl SF-200 gel filtration column (2.6 × 94 cm) equilibrated in buffer D (20% ethylene glycol, 40 mM NaCl, 80 mM sodium phosphate, pH 7.4). The enzyme was eluted with the same buffer at a flow rate of 25 ml/h. Fractions of 5 ml were collected and fractions with activity pooled. This preparation could be stored at 4 °C for several weeks without any detectable loss of activity.

Two gel filtration preparations were combined and concentrated with buffer exchange to buffer E (20% ethylene glycol, 20 mM Tris-HCl, pH 8.0) by ultrafiltration (PM-10 membrane). The resulting solution was applied to a strong anion exchange column (Resource Q, 6 ml; Pharmacia Biotech Inc.) equilibrated in buffer E and connected to an FPLC system (Pharmacia). The column was eluted at 1 ml/min with a gradient of 0–450 mM NaCl in buffer E as follows: 0–85 mM in 18 min, 85–110 mM in 10 min, 110–130 mM in 14 min, and 130–450 mM in 10 min. Fractions of 1 ml were collected. The activity of the main peak, which eluted at 120 mM NaCl, was collected and adjusted to pH 6.0 with 50 mM sodium phosphate.

The pooled fractions were loaded onto a Poros 20 SP strong cation exchange column (PerSeptive Biosystems, Cambridge, MA) equilibrated in buffer F (20 mM sodium phosphate, pH 6.0, 20% ethylene glycol) and eluted using the FPLC at a flow rate of 1 ml/min. Elution was carried out with a gradient of 0–500 mM NaCl in buffer F as follows: 125–225 mM in 40 min and 225–500 mM in 37 min. Fractions of 2 ml were collected. Two peaks of activity eluted: peak I at 210 mM and peak II at 225 mM NaCl. Peak II was dialyzed against 10 mM sodium phosphate, pH 7.2, containing 1 mM L-AA and the volume was reduced to 200 μl by lyophilization (Heto Lab Equipment, Lyngby, Denmark).

As a final step, the pooled fractions of peak II were separated by HPLC using a Zorbax gel filtration column GF-250 (9.4 × 250 mm) (Rockland Technologies Inc., Newport, DE) equilibrated in 750 mM NaCl, 50 mM sodium phosphate (pH 7.2). Fractions of 1 ml were collected at a flow rate of 1 ml/min.

Protein Determination—The protein concentration of extracts was determined according to Bradford (18) using bovine serum albumin as standard.

Determination of Molecular Mass—The molecular mass of the native GLDase was estimated by gel filtration on a Sephacryl SF-200 column (2.5 × 94 cm) equilibrated in 40 mM NaCl, 80 mM sodium phosphate (pH 7.4). The column was eluted at a flow rate of 20 ml/h and fractions of 4 ml were collected. The molecular mass was estimated by comparing the elution of GLDase with that of the standard proteins: ferritin (450 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.5 kDa).

SDS-PAGE—Analytical SDS-PAGE was performed in slab gels of 10% polyacrylamide as according to Chua (19). Proteins were visualized either by Coomassie Brilliant Blue R-250 staining (19) or silver nitrate staining (20).

Affinity Retardation Chromatography—Cytochrome *c* was covalently bound to thiol-activated Sepharose 4B as described by Azzi *et al.* (21) and packed into a column (1.0 × 20 cm) that eluted at flow rates of 8 ml/h in 10 mM sodium phosphate buffer (pH 7.4). Fractions of 2 ml were collected and tested for activity.

Lycorine Extraction—Lycorine was purified from non-flowering, whole plants of *Crinum jagus* or *Crinum asiaticum* as described by Davey *et al.*[2].

Partial Amino Acid Sequence Determination—Purified GLDase from the Poros 20 SP purification step was applied to SDS-PAGE. The separated polypeptides were blotted onto polyvinylidene difluoride membranes (Millipore) as described by Bauw *et al.* (23). NH_2-terminal and internal amino acid sequence analyses of the polyvinylidene difluoride-bound proteins were performed as described by Bauw *et al.* (24). Trypsin was used for the *in situ* digests and the resulting peptides were separated by reversed-phase HPLC. Amino acid sequencing was performed on a 473 protein sequencer (Applied Biosystems, Foster City, CA).

Isolation of Total RNA and First-strand cDNA Synthesis—Cauliflower floret tissue (300 mg) was ground to a powder in liquid nitrogen with a mortar and pestle and RNA was extracted using a method based on LiCl precipitation as described by Goormachtig *et al.* (25). The RNA isolated from cauliflower florets (4 μg) was used to synthesize first-strand cDNA according to the instruction manual for Superscript™ Preamplification System for first-strand cDNA synthesis (Life Technologies, Inc., Gaithersburg, MD).

Polymerase Chain Reaction—Degenerate oligonucleotides were synthesized on an oligonucleotide synthesizer (Applied Biosystems) and used as primers in polymerase chain reactions. The peptide sequences used for synthesizing the corresponding coding and complementary oligonucleotides were designed according to the partial amino acid sequence obtained earlier, and designated 3, 6, and 8 (*underlined* in Fig. 5).

First-strand cDNA synthesized from cauliflower florets was used as a template. The amplification mixture consisted of template, polymerase chain reaction buffer, 200–300 ng of each primer, 2.5 mM cNTP, and 1 unit of *Taq* polymerase in a total volume of 50 μl. The amplification program consisted of 32 cycles of denaturation (94 °C, 1 min), annealing (50 °C, 1 min), and primer extension (72 °C, 2 min). Products of the reaction were separated on 1% agarose gels, excised, and then purified according to the QIAEX Handbook (Diagen GmbH, Hilden, Germany). The purified products were cloned into a pGEM-T vector (Promega, Madison, WI).

Screening of cDNA Library—A cauliflower cDNA library constructed in λZAP II (Stratagene, La Jolla, CA) was used. Aliquots of the cDNA library were plated out using *Escherichia coli* XL-1 Blue cells on 23 × 23-cm baking plates (Nunc, Roskilde, Denmark) containing NZY agar. Approximately 600,000 plaques of the library were transferred onto duplicate nylon membranes (Hybond N$^+$; Amersham). The membranes were treated in accordance with the manufacturer's instructions for plaque blotting. DNA was fixed to membranes by irradiation with ultraviolet light (UV Stratalinker; Stratagene). A 250-bp polymerase chain reaction-amplified fragment was labeled with [α-^{32}P]dCTP using a random primed DNA labeling kit (Boehringer, Mannheim, Germany)

[2] M. W. Davey, G. Persiau, A. De Bruyn, J. Van Damme, G. Bauw, and M. Van Montagu, submitted for publication.

TABLE I
Purification scheme for GLDase
Mitochondrial extract from 15 kg of cauliflower florets were used for the preparation

Step	Volume	Protein	Activity		Fold	Recovery
			Total	Specific		
	ml	*mg*	*units*	*units/mg*		%
Acetone precipitation	2,500	1,510	44,900	30	1	100
DEAE ion exchange	83	55	46,500	845	28	104
Phenyl-Sepharose	38	21	30,800	1,467	49	69
Gel filtration	54	11	20,900	1,900	63	47
FPLC Resource Q	32	0.3	8,100	27,000	900	18
FPLC Poros 20 SP	4	0.01	508	50,800	1,693	1.1

and subsequently used as probe for screening the cDNA library. The membranes were washed for 4 h at 65 °C in hybridization buffer (1% (w/v) bovine serum albumin, 7% (w/v) SDS, 1 mM EDTA, and 0.25 M sodium phosphate, pH 7.2), before 20 h incubation with the ^{32}P-labeled probe in hybridization buffer at 65 °C. The membranes were then rinsed twice for 15 min with 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na_3-citrate, pH 7.0) and 1% SDS at room temperature and exposed to X-Omat AR film (Kodak, Rochester, NY) with an enhancer screen for autoradiography. Plaque-purified phage clones were converted into phagemids (Bluescript SK-/+; Stratagene) by *in vivo* excision using the ExAssist™ System.

DNA Sequence Determinations—DNA sequence determinations were carried out in accordance with protocols obtained from Applied Biosystems. Initial sequences were obtained by use of T7 and T3 vector primers. To complete the sequences on both strands, cDNA-specific primers were used. The sequence analyses were carried out using software of the Genetics Computer Group (Madison, WI).

Expression in Yeast—To express the GLDase cDNA in yeast (*Saccharomyces cerevisiae*), the Bluescript vector containing the full-length cDNA was digested with *ApaI* and *KpnI* and a 27-bp adapter containing a *NotI* restriction site subsequently ligated into the *ApaI-KpnI*-linearized vector. The resulting construct containing two *NotI* restriction sites was cloned into the *NotI* restriction sites of the pFL61 vector (26). Yeast cells of the strain W303B (*Matα, ade2, ura3, his3, trp1, leu2, can*1-100) (27) were transformed by the method of Dohmen *et al.* (28) and plated on selective 1.5% agar plates (lacking uracil) containing minimal SD medium (0.2% yeast nitrogen base (Difco, Detroit, MI), 0.7% ammonium sulfate, 2.7% glucose) supplemented with adenine, tryptophan, leucine at 20 μg/ml, and histidine at 10 μg/ml (as above minus agar). Transformed cells were transferred to liquid SD medium and grown for 3 days at 30 °C. The cells were collected by centrifugation (8,000 × *g*, 15 min), washed, and resuspended in 50 mM Tris-HCl (pH 8.0). For GLDase activity tests and protein determinations the cells were disrupted by two passages through a French Press after a cycle of freezing (−70 °C) and thawing.

Extraction and HPLC Analysis of L-GL—Up to 1 g of plant tissue was first thoroughly homogenized using a pestle and mortar in liquid nitrogen, and extracted using 10% trichloroacetic acid to precipitate proteins and inhibit degradative enzymes. After filtration and partitioning against water-saturated diethyl ether to remove trichloroacetic acid, samples were concentrated and injected onto the C18 HPLC column, eluted with 0.1% trifluoroacetic acid. Peaks eluting in the region of L-GL elution were collected and tested for their ability to serve as a substrate for GLDase. Analogous peaks from up to 10 runs were combined, dried under vacuum, and re-injected onto the aminopropyl HPLC column for weak anion exchange. Once again peaks eluting in the region of the L-GL standard were collected and tested for their ability to serve as a substrate for GLDase. Positive peaks from several runs were pooled, concentrated, and finally reinjected on a C18 reversed-phase HPLC column eluted with phosphoric acid (pH 2.5). HPLC was carried out using a 600E pump (Waters, Milford, MA) and a Waters 996 diode-array detector. Injections (20–40 μl) were made using a WISP 412 (Waters) autosampler onto a C18, 3-μM spherical particle size, 250 × 4.6 mm inner diameter, reversed-phase HPLC column (Bio-Rad), fitted with a 10-mm guard column. Separations were carried out isocratically at 800 μl/min with phosphoric acid (pH 2.5), or 0.1% trifluoroacetic acid as mobile phase. Data were collected and analyzed, and the entire system was controlled using the Millenium 2010 (v1.15) chromatography management system (Waters). Weak anion exchange separations were carried on a 250 × 3.6-mm aminopropyl column (Phenomenex Inc., Torrance, CA), eluted isocratically with 15% (v/v) 20 mM KH_2PO_4 (pH 6.0), in acetonitrile. The column was regenerated after each analysis with a 10-min linear gradient of 15–50% acetonitrile in 20 mM KH_2PO_4 (pH 6.0) at 1 ml/min. Strong anion exchange HPLC with pulsed amperometric electrochemical detection was carried out on the same system fitted with an HP 1049A electrochemical detector containing a gold amalgam-working electrode at an operating potential of +100 mV. Separations were performed on a 300 × 4.6-mm, Dionex PA-100 strong anion exchange column (Dionex Corp., Sunnyvale, CA) eluted with a 20-min linear gradient of 0 to 200 mM sodium acetate in 3 ml/liter NaOH.

RESULTS

Enzyme Purification

A summary of the purification of GLDase from cauliflower florets is presented in Table I. As the enzymatic activity was found to be most stable in 20% ethylene glycol this reagent was included in all buffers except for buffers A and B used in the first two purification steps. Interestingly, after the DEAE-Sepharose step the total GLDase activity increased slightly, probably due to removal of inhibitory compounds present in the crude extract. The first three purification steps had relatively little influence on the purity of GLDase, but the FPLC Resource step (strong anion exchange) resulted in an increase in the purification factor from 63 to 900, although there was a corresponding decrease in recoveries to only 47% compared with the activity present in the gel filtration pool. After passage through the strong cation exchange column (Poros 20 SP), GLDase activity was resolved into two peaks designated I and II (Fig. 1). The activity forming the latter peak was used for further analysis. At this stage GLDase was purified 1693-fold from the initial mitochondrial fraction with a recovery of 1.1% (Table I). The purity of the final enzyme preparation was confirmed by SDS-PAGE, where we consistently obtained three polypeptide bands corresponding to approximately 56, 30, and 26 kDa (Fig. 2). Further purification of the enzyme by a high resolution gel filtration on a Zorbax GF 250 column did not result in elimination of the 30- and 26-kDa polypeptide bands; and subsequent amino acid sequence analyses revealed them to be breakdown products of the 56-kDa band. The native molecular mass of the enzyme was estimated to be approximately 56 kDa by Sephacryl SF-200 (Fig. 3) and Zorbax GF 250 high resolution gel filtration.

Partial Amino Acid Sequence Determination of Purified GLDase Polypeptides

NH_2-terminal sequence analysis of the complete 56- and 30-kDa polypeptide bands were found to be identical, and the partially determined sequence of the 26-kDa band was located within the deduced amino acid sequence of the GLDase cDNA (Asp-273 to Leu-289). Trypsin digestions of the 56-kDa protein yielded a series of peptides which were separated by reversed-phase HPLC. A number of the peptides were subjected to partial sequence analysis and could again be located in the GLDase cDNA, as indicated in Fig. 5.

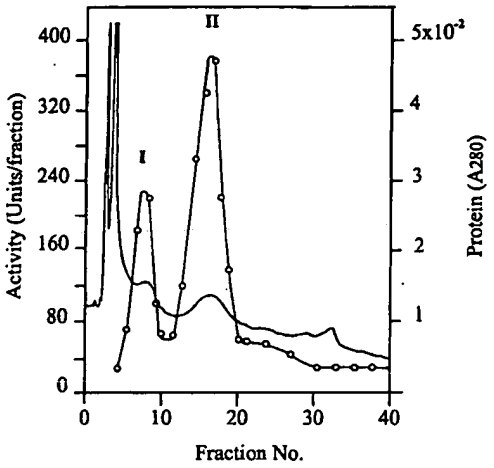

FIG. 1. **Separation and purification of GLDase activity (○) peak I and peak II by a Poros SP cation exchange column.** Protein (A_{280}) (—).

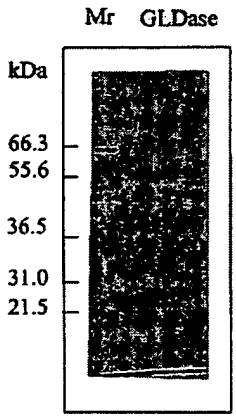

FIG. 2. **SDS-PAGE.** *Lane A*, molecular mass standards; *lane B*, GLDase peak II from the Poros SP (strong anion exchange) purification step, analyzed by SDS-PAGE after an additional high-resolution HPLC gel filtration step. A polypeptide band corresponding to approximately 56 kDa (GLDase), and two degradation products of 30 and 26 kDa (confirmed by amino acid sequence analyses) were visualized by silver nitrate staining.

Characterization

Substrate Specificity and pH Dependence—Various isomeric compounds were tested as possible substrates for the purified GLDase using cytochrome *c* as electron acceptor. These were L-GL, D-galactono-γ-lactone, D-gulono-γ-lactone, L-GuL, D-erythronic-γ-lactone, D-xylonic-γ-lactone, L-mannono-γ-lactone, D-galactonic acid, D-glucuronic acid, and D-gluconic acid. Apart from L-GL, none of the compounds tested could serve as a substrate for GLDase because no reduction of cytochrome *c* was observed.

GLDase obeyed Michaelis-Menten-type kinetics using L-GL as substrate. With the method of Lineweaver and Burk (Fig. 4), the K_m value was determined to be 3.3 mM with a V_{max} of 7.1 units/min. Concentrations of L-GL used were from 1.0 to 32.6 mM. Substrate inhibition was observed at 32.6 mM.

The pH dependence of the enzyme activity was examined using 50 mM sodium phosphate buffer in the pH range from 6.0 to 7.6 and 50 and 100 mM Tris-HCl in the range between 7.4 and 8.8 at 22 °C with 4.2 mM L-GL. A broad maximum of activity between pH 8.0 and 8.5 was observed (results not shown).

Electron Acceptors—The enzyme assay is based on the reduction of cytochrome *c* by GLDase, in which for each micromole of oxidized L-GL, 2 μmol of cytochrome *c* are reduced, because the L-AA formed is spontaneously oxidized by cytochrome *c* to dehydroascorbic acid. The purified GLDase showed strict specificity for cytochrome *c*, and neither FAD, NAD, NADP, nor molecular oxygen were able to serve as electron acceptors for the enzyme.

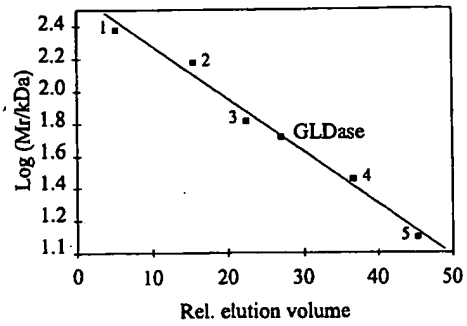

FIG. 3. **Estimation of molecular mass of GLDase.** The native molecular mass was estimated by gel filtration chromatography on Sephacryl SF-200. The *arrow* indicates GLDase activity. Molecular mass standards used were: *1*) ferritin (450 kDa); *2*) alcohol dehydrogenase (150 kDa); *3*) bovine serum albumin (66 kDa); *4*) carbonic anhydrase (29 kDa); and *5*) cytochrome *c* (12.5 kDa).

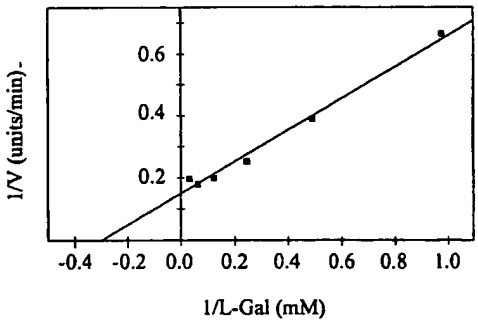

FIG. 4. **Lineweaver-Burk plot of GLDase activity as a function of L-galactono-γ-lactone concentration.**

Inhibitors/Stimulators—The effect of various substrate analogues, organic inhibitors, and some divalent metal ions were examined for their influence on the enzyme activity. The oxidation of L-GL by GLDase was tested in the presence of equimolar concentrations of each of the following compounds: D-galactono-γ-lactone, D-gulono-γ-lactone, L-gulono-γ-lactone, D-erythronic-γ-lactone, D-xylonic-γ-lactone, L-mannono-γ-lactone, D-galactonic acid, D-glucuronic acid, and D-gluconic acid. None of these had any influence on the reaction rate.

Of the divalent metal salts we tested, $MgCl_2$, $CaCl_2$, and $SrCl_2$ had no effect on the GLDase activity at concentrations up to 15 mM. The chelating agent EDTA had no significant effect on the enzyme activity supporting the conclusion that there was no metal requirement for the enzymatic activity.

Sulfhydryl-modifying agents, however, were able to partially inhibit GLDase: *N*-ethylmaleimide, monoiodoacetic acid, and *p*-hydroxymercuribenzoic acid inhibited the enzyme activity by 18% at 12.5 mM, 42% at 26.9 mM, and 81% at 0.4 mM, respectively. These observations indicate that cysteine residues play an important role in the enzyme catalysis. We did not observe any inhibition of the GLDase-dependent reduction of cytochrome *c* in the presence of 5.2 mM riboflavin, a well known flavoprotein inhibitor (29–31).

Lycorine, an alkaloid isolated from members of the *Amaryllidaceae* has been reported to be a specific inhibitor of ascorbic acid biosynthesis in plants and animals at concentrations as

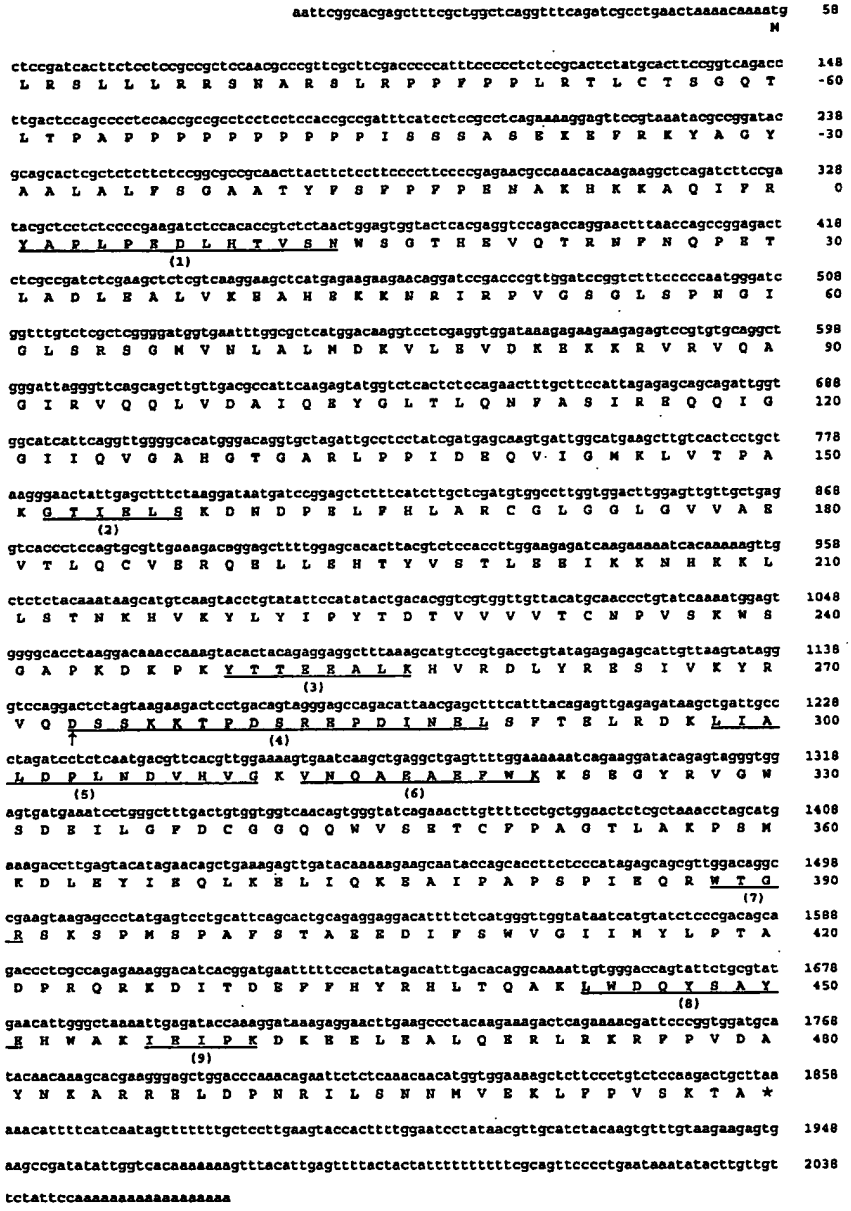

FIG. 5. **Nucleotide sequence and predicted amino acid sequence of GLDase.** Nucleotides are numbered from the first base of the cDNA insert. The deduced amino acid sequence is indicated below the nucleotide sequence in single-letter code. The first methionine of the open reading frame is designated as the first amino acid of the putative polypeptide. The termination codon is indicated by an *asterisk*. Amino acid sequences determined from GLDase polypeptides are *underlined* and *numbered 1–9*. Degenerate oligonucleotides were designed based on peptides 3, 6, and 8. "↕" indicates the point of breakage forming the 26- and 30-kDa degradation products separated by SDS-PAGE (Fig. 2).

low as 1 μM (32–34); once again, however, no influence of lycorine on GLDase activity could be found at concentrations of up to 100 μM.

Cytochrome c Affinity Chromatography—Partly purified enzyme extract was observed to be slightly retarded compared with other proteins (measured as the absorption at 280 nm) when eluted from a cytochrome *c* affinity column. This indicated interaction between GLDase and cytochrome *c*.

Isolation and Sequencing of GLDase cDNA Clone

DNA fragments were obtained by polymerase chain reaction amplification of oligo(dT)-primed cDNA using degenerate oligonucleotides (based on the peptide sequences) as primers. These DNA fragments were subcloned into a pGEM-T vector and sequenced. One 400-bp fragment contained a nucleotide sequence which corresponded to the amino acid sequence of one of the sequenced internal peptides in addition to the sequences corresponding to the primers. Therefore, this fragment was radiolabeled and used as a probe to screen a cDNA library from cauliflower. We screened 2×10^6 plaques resulting in isolation of several positive clones. After *in vivo* excision of the Bluescript plasmid followed by digestion with *Eco*I and *Kpn*I, the two longest cDNA inserts were found to be approximately 2,000 bp. Subsequent subcloning and sequencing revealed an uninterrupted open reading frame of 1803 nucleotides, containing all of the partially sequenced tryptic peptides, the NH_2-terminal amino acid sequence, the first ATG codon (position 56) representing the consensus sequence of an initiator codon (35), and a TAA terminator codon. The presence of these elements showed that the full-length cDNA corresponding to the purified protein had been isolated. Fig. 5 shows the deduced amino acid sequences of the 1803-bp open reading frame coding for 600 amino acids, a 55-bp putative 5′-noncoding region, and a 206-bp 3′-noncoding region including a poly(A) tail. A hexanucleotide AATAAA consensus signal for polyadenylation is found 20 nucleotides before the poly(A)$^+$ tract. Interestingly, nucleotides coding for the determined NH_2-terminal amino acid sequence were found 270 bp downstream from the initiator codon, indicating that the protein is synthesized as a preprotein (600 amino acids with a predicted molecular mass of

67,829 Da). The resulting mature protein of 509 amino acids has a calculated molecular mass of 57,837 Da and a theoretical pI value of 6.85. A putative mitochondrial signal is also present (36).

Expression in Yeast

The GLDase cDNA was cloned into a pFL61 yeast vector (26) in both the sense and antisense orientations relative to the phosphoglycerate kinase promoter and terminator. Untransformed and transformed yeasts were grown and extracts were prepared and tested for GLDase activity. Extracts from yeast transformed with a sense-oriented GLDase cDNA showed a specific GLDase activity of 3.0 units/min/mg protein compared with those made from extracts from untransformed yeast and yeast transformed with antisense orientated GLDase cDNA in which no GLDase activity could be measured with L-GL as substrate (Fig. 6).

HPLC Analysis of L-GL

We used several different systems for the analysis of L-GL by HPLC. These included ion suppression reversed-phase HPLC, weak anion exchange HPLC, and strong anion exchange HPLC. In no case was it possible to obtain unequivocal resolution of L-GL from all other sugar-lactone analogues, but semi-preparative separations using weak anion exchange and reversed-phase HPLC in combination with spectrophotometric assays for GLDase activity, allowed us to consistently identify a fraction that co-migrated with L-GL standard and which served as a substrate for the GLDase-based reduction of cytochrome *c*. Peaks co-eluting in all three systems with L-GL standard were found to be able to serve as a substrate for GLDase (results not shown). This indirect evidence strongly suggests the presence of a natural substrate for GLDase in plant tissue extracts. In addition to this observation, acid extracts of plant tissues were resolved using pulsed amperometric detection and strong anion exchange on a Dionex PA-100 column. Under conditions of high pH (pH 11–12), it is possible to ionize neutral carbohydrates at the C-2 OH position, allowing the separation on appropriate ion exchange columns. Analysis of acid extracts from cauliflower and parsley by strong anion exchange HPLC with pulsed-amperometric detection at a gold electrode showed the presence of small amounts of a peak that co-migrated with L-GL (data not shown). However, in this system, L-GL also co-migrates with DL-GuL and with D-GL, so that it is not possible to unequivocally demonstrate the presence of this compound as a natural substrate.

DISCUSSION

GLDase was purified 1693-fold from cauliflower florets by a 5-step method with 1.1% recovery. The loss in recovery was approximately 20% in each purification step. This compares favorably with the results of Ôba *et al.* (14) who recently published a 5-step purification of GLDase from sweet potato roots in which the enzyme was purified 294-fold with a recovery of 0.9% with a specific activity of 37,000 units/mg. By comparison, after our purification method we obtained a specific activity of 50,800 units/mg.

From the Poros SP column (strong anion exchange) GLDase activity was separated into two peaks of activity (I and II), suggesting the existence of at least two isoforms of GLDase. The most pure and abundant peak, peak II, was subjected to high-resolution gel filtration by HPLC and analyzed by SDS-PAGE (Fig. 2). A polypeptide band corresponding to approximately 56 kDa and two degradation products (confirmed by amino acid sequence analysis) of 30 and 26 kDa separated on the gel.

In most respects, the physical characteristics of GLDase from cauliflower are similar to those of the enzyme purified from sweet potato roots. With regard to the substrate specificity we found like Mapson and Breslow (12) that GLDase was absolutely specific for L-GL; Ôba *et al.* (14), however, observed a 1% oxidation of L-GuL relative to L-GL by the sweet potato enzyme. We measured the K_m value of GLDase for L-GL as substrate to be 3.3 mM which is again in the same range as the value obtained by Mapson and Breslow (12), but considerably higher than the value of 0.12 mM obtained by Ôba *et al.* (14).

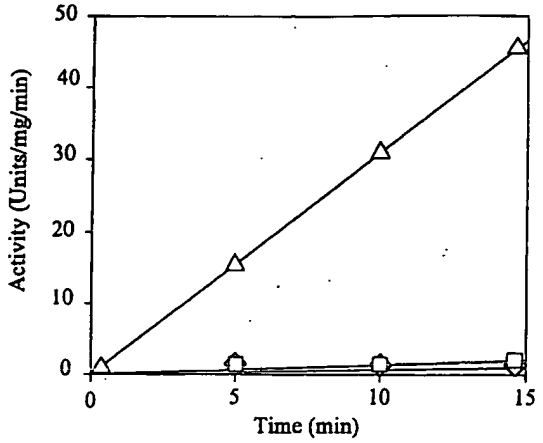

FIG. 6. **Expression in yeast.** GLDase activity in nontransformed yeast (□), and in yeast transformed with the GLDase in sense orientation (△), and in antisense orientation (◇).

However, the native molecular mass of 56 kDa determined by gel filtration is identical to the value obtained by Ôba *et al.* (14). According to our experiments, GLDase has a pH optimum between 8.0 and 8.5, which again corresponds well with the results obtained by Mapson and Breslow (12) and Ôba *et al.* (14).

The GLDase enzyme from cauliflower seems to require sulfhydryl groups for its activity, as reduced activity was observed in the presence of reagents which inactivate these groups. Strongest inhibition was observed with 0.4 mM *p*-hydroxymercuribenzoic acid which caused 81% inhibition. These observations are in accordance with results obtained by Mapson and Breslow (12), who obtained 50% or more inhibition with all sulfhydryl group-modifying agents tested.

Arrigoni *et al.* (37) recently published results from which they concluded that the alkaloid lycorine acts by inhibiting the conversion of L-GL to L-AA. Consequently, the enzyme we have purified from cauliflower is different to the homologous enzyme which Arrigoni *et al.* (37) used for their measurements. These authors also isolated GLDase activity from cauliflower using a different protocol including detergent. We were unable to detect any influence of lycorine on the activity of GLDase from cauliflower at concentrations of up to 100 μM lycorine.

Based on the partial amino acid sequences of tryptic peptides, the cDNA for GLDase was cloned and characterized. The complete amino acid sequence deduced from the cDNA and the localization of the NH_2-terminal amino acid sequence suggest that the mature GLDase protein is preceded by a 91-amino acid pre-peptide. We consider GLDase from cauliflower to be a mitochondrial enzyme as it was purified from a mitochondrially enriched extract from cauliflower florets. This corresponds well with the fact that the deduced pre-protein contains a relatively high number of Ala, Leu, Arg, and Ser residues (11, 10, 8, and 10, respectively); and relatively few Asp, Glu, Ile, and Val residues (0, 3, 2, and 0, respectively), which is characteristic for polypeptides targeted to the mitochondria (38, 39). In addition, the GLDase pre-protein cleavage site FR↓YA resembles a

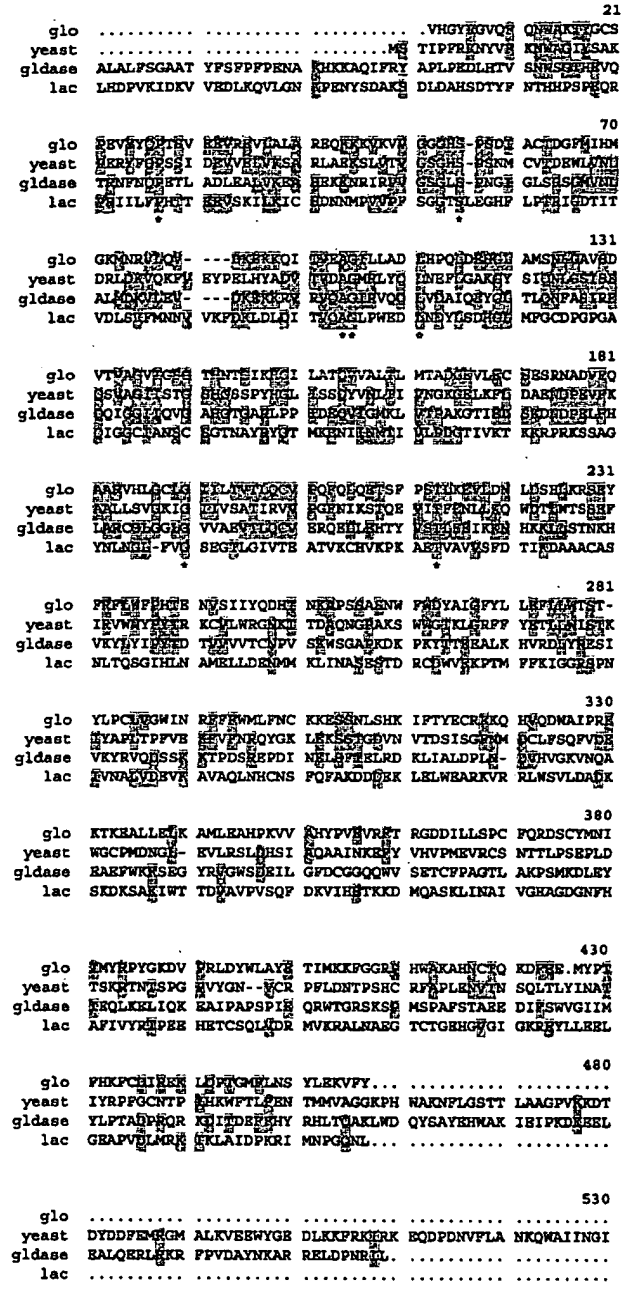

FIG. 7. **Sequence comparison.** Alignment of the amino acid sequence (residue −29 to 509) of GLDase (*gldase*) with the amino acid sequence of L-gulono-γ-lactone oxidase from rat (*glo*) (48), a sequence from the *S. cerevisiae* sequencing project (yeast),[3] and D-lactate ferricytochrome *c* oxidoreductase from *S. cerevisiae* (*lac*) (22). *Dashes* represent regions where the sequences have been extended to allow optimal sequence alignment. Identical residues are indicated by *shaded letters*. *Asterisks* indicate identical residues present in all four sequences. Indicated *numbers* on the *right* refer to the amino acid positions used for GLDase in Fig. 5.

cleavage site motif (RXY ↓ (S/A) which is relatively common in a number of higher and lower eukaryotes (36). These data are in accordance with results obtained by Ôba *et al.* (13) who by sucrose density gradient cell fractionation of extract from potato tuber tissue detected GLDase activity in the same fractions as fumarase, a mitochondrial marker enzyme. By the same technique, Mutsuda *et al.* (40) judged the enzyme to be located in mitochondrial membranes of spinach leaves. The relative molecular mass of GLDase was predicted to be 57,837 Da from the cDNA, which corresponds well with the molecular mass of 56 kDa, estimated by SDS-PAGE and gel filtration. We found one potential *N*-glycosylation site Asn-*X*-Ser/Thr at position 13 in the mature protein, but the enzyme was unretarded on a lectin concanavalin A column, which binds molecules containing an α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related sugars (41).

It has been suggested that GLDase contains a covalently bound flavin moiety as prosthetic group, because inhibition of GLDase activity by flavoprotein inhibitors was reported by Mapson and Breslow (12) and Ôba *et al.* (14). However, we could not observe any inhibition of GLDase from cauliflower by riboflavin or obtain the typical flavin protein absorption spectrum from the purified GLDase. In addition, there was no significant homology between the deduced amino acid sequence of cauliflower GLDase and the sequences of flavin-binding regions of the following flavoenzymes: rat liver dimethylglycine dehydrogenase (42), beef heart succinate dehydrogenase (43), *E. coli* succinate dehydrogenase (44), *E. coli* fumarate reductase (45), *Arthrobacter oxidans* 6-hydroxy-D-nicotine oxidase (46), and rat liver NADPH-cytochrome P-450 oxidoreductase (47).

Interestingly, an homology search with cauliflower GLDase in protein data bases revealed that the first 230 NH_2-terminal amino acid of GLDase had 28% identity with an NH_2-terminal stretch of L-gulono-γ-lactone oxidase (EC 1.1.3.8) from rat (48), 26% with a yeast clone,[3] and 16% with an NH_2-terminal stretch of D-lactate ferricytochrome *c* oxidoreductase from yeast (22) (Fig. 7). This conservation between the NH_2-terminal parts of L-gulono-γ-lactone oxidase and GLDase could indicate that this domain is involved in similar functional roles.

Here, we have clearly shown that the isolated GLDase is 100% specific for L-GL and that a peak that co-elutes with L-GL in four different HPLC separation systems, and can serve as a substrate for GLDase, is naturally present in plant extracts. Although we cannot unequivocally rule out the presence of some other compound, the high substrate specificity of GLDase strongly suggests that L-GL is a natural constituent of plant tissues and that this pathway of L-AA biosynthesis is physiologically relevant.

The results presented here are the first important step in the characterization of the biosynthetic pathway of L-AA in plants. In our laboratory we are progressing toward making plants transformed with the GLDase cDNA, which will allow us to down-regulate the expression of GLDase by antisense technology and study the role of L-AA in plants. Furthermore, in the near future the isolation of the GLDase cDNA may give us the possibility to engineer crops containing stably increased levels of vitamin C.

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Purification and Characterization of Electron-transfer Flavoprotein:Rhodoquinone Oxidoreductase from Anaerobic Mitochondria of the Adult Parasitic Nematode, *Ascaris suum**

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Electron-transfer flavoprotein:rhodoquinone oxidoreductase (ETF-RO) was purified to homogeneity from anaerobic mitochondria of the parasitic nematode, *Ascaris suum*. The enzyme has a subunit molecular mass of 64.5 kDa and is similar in many respects to the lectron-transfer flavoprotein:ubiquinone oxidoreductase (ETF-UO) characterized in mammalian tissues. EPR spectroscopy of the purified enzyme revealed signals at $g = 2.076$, 1.936, and 1.883, arising from an iron-sulfur center, as well as signals attributable to a flavin semiquinone. Potentiometric titration on the enzyme with dithionite yielded an oxidation-reduction midpoint potential (E_m) for the iron-sulfur center of +25 mV at pH 7.4. The reduction of flavin occurred in two distinct steps, with a flavin semiquinone radical d tected as an intermediate. The E_m values for the two steps in the complete reduction of flavin were +15 mV and −9 mV, respectively.

Physiologically, the ascarid ETF-RO accepts electrons from a low potential quinone, rhodoquinone, and functions in a direction opposite to that of the ETF-UO. Incubations of *A. suum* submitochondrial particles with NADH, 2-methylcrotonyl-CoA, purified *A. suum* electron-transfer flavoprotein and 2-methyl branched-chain enoyl-CoA reductase resulted in significant 2-methylbutyryl-CoA formation, which was inhibited by both rotenone and antisera to the purified ETF-RO. Quinone extraction of the submitochondrial particles with dry pentane resulted in almost the complete loss of 2-MBCoA formation by the system. However, the reincorporation of rhodoquinone, but not ubiquinone, restored over 50% of the NADH-dependent 2-MBCoA formation.

Mitochondrial energy metabolism in muscle of the adult parasitic nematode, *Ascaris suum*, is anaerobic and results in the accumulation of the novel branched-chain fatty acids, 2-methylbutyrate and 2-methylvalerate (1, 2). Ascarid mitochondria lack a functional tricarboxylic acid cycle and electron-transport is cyanide-insensitive (3, 4). However, the NADH-dependent reductions of fumarate and apparently 2-methyl branched-chain enoyl-CoAs are coupled to site 1, electron-transport associated, ADP phosphorylations (4, 5). 2-Methylbutyrate and 2-methylvalerate are formed by the condensation of an acetyl-CoA and a propionyl-CoA or two propionyl-CoAs, respectively, with the subsequent condensation of the products through a series of reactions similar to a reversal of β-oxidation (6, 7). The final reaction in this pathway, the NADH-dependent reduction of 2-methylcrotonyl-CoA or 2-methyl-2-pentenoyl-CoA requires *A. suum* submitochondrial particles and two soluble flavoproteins, electron-transfer flavoprotein (ETF)[1] and 2-methyl branched-chain enoyl-CoA reductase, which previously have been purified to homogeneity and characterized (5, 8).

ETF:ubiquinone oxidoreductase (ETF-UO) is a membrane-bound, iron-sulfur flavoprotein (9, 10). In mammalian mitochondria, it plays an important role in the β-oxidation of fatty acids and oxidative demethylation reactions by shuttling reducing equivalents from a soluble ETF to ubiquinone of the electron-transport chain (13). In adult *A. suum* mitochondria, ETF:rhodoquinone oxidoreductase (ETF-RO) functions in the opposite direction and couples rotenone-sensitive NADH oxidation with ETF and the subsequent reduction of 2-methyl branched-chain enoyl-CoAs (5, 8). Adult *A. suum* muscle mitochondria are modified by the presence of a low potential quinone, rhodoquinone ($E_m = -64$ mV), instead of ubiquinone ($E_m = +110$ mV). In addition, the redox potential of the *b* cytochrome associated with the fumarate reductase appears to be more negative than that of the corresponding mammalian succinic dehydrogenase, facilitating electron-flow in the reverse direction (14, 15). It is well documented that the reactions from acyl-CoA dehydrogenase to ubiquinone in mammalian mitochondria are potentially reversible depending on the NADH/NAD^+ ratio (11). NADH/NAD^+ ratios appear to be dramatically elevated in adult ascarid mitochondria (12, 13). Therefore, the present study was designed to purify ETF-RO activity from membranes of adult *A. suum* muscle mitochondria to assess its role in the substantial NADH-dependent 2-methyl branched-chain enoyl-CoA reduction catalyzed by these organelles.

EXPERIMENTAL PROCEDURES

Materials—CoA and CoA esters were obtained from P-L Biochemicals. 2-Methylbutyrate was from Aldrich. DEAE-Bio-Gel and Bio-Gel HT were from Bio-Rad. All other reagents were obtained from commercial sources and were the highest purity available. 2-Methybutyryl-CoA was synthesized by way of its L-acylimidizole and puri-

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[1] The abbreviations used are: ETF, electron-transfer flavoprotein; DPB, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone; E_m, oxidation-reduction midpoint potential; ETF-RO, electron-transfer flavoprotein: rhodoquinone oxidoreductase; ETF-UO, electron-transfer flavoprotein: ubiquinone oxidoreductase; 2-MBCoA, 2-methylbutyryl-CoA; PAGE, polyacrylamide gel electrophoresis; SMP, submitochondrial particles.

fied by high pressure liquid chromatography as described previously (16). 2,3-Dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone (DPB) and antisera to the beef heart ETF-UO were a generous gift of Dr. Brian Ackrell, University of California, San Francisco. Ubiquinone-1, a product of Eisai, Tokyo was a gift of Dr. Shinzaboro Takamiya, Juntendo University, Tokyo. ETF and 2-methyl branched-chain enoyl-CoA reductase were purified to apparent homogeneity from isolated *A. suum* mitochondria, as described previously (5, 8).

Assay of ETF:Rhodoquinone Oxidoreductase Activity—ETF-RO activity was assayed spectrophotometrically by following a decrease in absorbance at 282 nm using DPB, a synthetic analog of Coenzyme Q, as a terminal electron acceptor (17). The incubation mixture contained 10 mM Tris-HCl, pH 7.4, 25 μM 2-MBCoA, 0.5 μM ETF, 1 μM 2-methyl branched-chain enoyl-CoA reductase, 60 μM DPB, and enzyme in a final volume of 1 ml. The measurement of DPB reduction at 282 nm is complicated by a increase in absorbance due to the formation of 2-methylcrotonyl-CoA. Therefore, an extinction coefficient appropriate for the assay was calculated as described by Ramsay *et al.* (18), using an extinction coefficient of 16.9 mM^{-1} cm^{-1} for DPB (17). The corrected value was 12.6 mM^{-1} cm^{-1}. This assay was useful for assaying ETF-RO activity in column fractions and for determinations of enzyme stability. The assay was linear up to 60 nM ETF-RO. Activity was absolutely dependent on the presence of both coupling enzymes, and both were present at saturating concentrations. Similar assays using nitroblue tetrazolium, instead of DPB, as described for the pig liver ETF-UO, were not proportional to enzyme concentration (10).

Isolation of ETF-RO—Muscle strips were obtained from adult female *A. suum* by dissection, and mitochondria were isolated and stored at −20 °C as described previously (8). ETF-RO activity was isolated using procedures described for the isolation of ETF-UO from pig liver mitochondria with modifications (10). Frozen mitochondria were thawed, centrifuged at 10,000 × *g* for 10 min, and the pellet was resuspended in 240 mM sucrose, 10 mM Tris-HCl, 2 mM EGTA, 1 mM dithiothreitol, 5 mM potassium succinate, pH 7.5, to about 10 mg protein/ml. The resuspended mitochondria were extracted with cholate (0.18 mg/mg protein) for 20 min in an ice bath, and the mitochondrial membranes were sedimented at 100,000 × *g* for 3 h. The pellet was resuspended in the above buffer and again extracted with cholate (0.25 mg/mg protein). The cholate extract was centrifuged at 100,000 × *g* for 1 h, and the supernatant fraction was immediately fractionated with solid ammonium sulfate. Protein precipitating between 40 and 60% saturation was centrifuged at 10,000 × *g* for 30 min, and the floating precipitate was dissolved in a minimal volume of 10 mM Tris-HCl, pH 7.5, containing 0.1% (w/v) Triton X-100. This fraction, which contained most of the ETF-RO activity was dialyzed against the same buffer for 3 h and centrifuged at 100,000 × g for 1 h. The supernatant fraction was applied to a Sephadex G-100 column previously equilibrated with dialysis buffer. The yellow-green fraction eluting with the void volume was immediately applied to a DEAE-Bio-Gel column (8 × 1.8 cm) equilibrated with 10 mM Tris-HCl, 0.1% (w/v) Triton X-100, pH 7.5. The column was washed with 100 mM Tris-HCl, pH 7.5, and the ETF-RO activity was eluted with a linear gradient of 100–220 mM Tris-HCl, 0.1% (w/v) Triton X-100, pH 7.5, at about 150 mM Tris-HCl. Fractions containing ETF-RO activity were pooled and concentrated to at least 1 mg/ml in an Amicon stirred cell with a PM-30 membrane. The concentrated ETF-RO could be stored at −20 °C in 50% glycerol for 1 month with little change in activity or spectral properties. Use of an Amicon Centricon-30 microconcentrator resulted in significant binding of the purified enzyme to the membrane and associated loss of activity. After chromatography on DEAE-Bio-Gel, most preparations on ETF-RO yielded single bands after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Preparations not homogenous were further purified by chromatography on hydroxylapatite as described by Beckman and Frerman (10) with about a 50% loss of ETF-RO activity, only about 20% of which was restored by the addition of FAD to the assay mixture.

Methods—Iron was determined by the method of Beinert (19), acid labile sulfide by the procedure described by King and Morris (20), and flavin according to Siegel (21). Protein was determined by the method of Lowry *et al.* (22), using bovine serum albumin as standard.

Amino Acid Analysis—Samples were analyzed in a Dionex D300 Amino Acid Analyzer equipped with a Dionex DC 5A column (Na^+ resin) following hydrolysis at 110 °C in 6 N HCl in sealed, evacuated tubes for 24, 48, and 72 h. Threonine and serine destruction were corrected for by extrapolation to "zero time." For amino terminal sequencing, samples were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to ProBlott in 3-(cyclohexylamino)-1-propanesulfonic acid and 10% methanol, pH 11, for 30 min (23). ProBlott was removed from the transblotting sandwich and thoroughly rinsed with water. The ProBlott then was saturated in 100% methanol for a few seconds and stained for 1 min with 0.1% Coomassie Brilliant Blue R-250, 40% methanol, 1% (v/v) acetic acid, destained in 50% methanol, and extensively rinsed in water. After excision the protein was sequenced on an Applied Biosystems model 477 A protein sequencer with on-line determination of phenylthiohydantoin derivatives.

Preparation of Antibodies—Two-month old, male, New Zealand White rabbits were injected subdermally with a total of 250 μg of the purified ETF-RO in complete Freund's adjuvant, as described previously (24). Three weeks later the rabbits were boosted intraperitoneally with 100 μg of the enzyme in 10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl (PBS). One week later, the rabbits were bled from the marginal ear vein, and IgG fractions were prepared. Serum was routinely collected at 1-month intervals, 1 week after a boost with 100 μg protein. Immunoglobins were prepared by double ammonium sulfate precipitation at 33% saturation, dialysis against 20 mM sodium phosphate buffer, pH 6.3, and chromatography on DEAE-cellulose. The protein peak that emerged at the void volume was concentrated by ultrafiltration, dialyzed overnight against 100 mM sodium borate, pH 8.2, 150 mM NaCl, and stored at −70 °C.

Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed in 10% gels, essentially as described by Laemmli (25). Gels were stained for 2 h in 0.1% Coomassie Brilliant Blue and destained in 7% acetic acid, 7% 2-propanol. Molecular weight standards were phosphorylase *b*, 97,400, bovine serum albumin, 66,200, ovalbumin, 42,699, soybean trypsin inhibitor, 21,500, and lysozyme, 14,400.

For immunoblotting, samples were transferred to nitrocellulose overnight and treated as described previously (24). The sequence of washing and blocking steps was as follows: one 45-min wash in Tris-buffered saline (TBS) containing 4% bovine serum albumin and 0.1% Tween-20; one 120-min incubation with the first antibody diluted 1:1000 in TBS containing 4% bovine serum albumin and 0.1% Tween-20; three 20-min washes in TBS; one 60-min incubation with goat-antirabbit IgG conjugated to alkaline phosphatase, diluted (1:7500) in TBS containing 0.1% Tween-20; three 10-min washes in TBS with 0.1% Tween-20; and finally color development in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl, 5 mM $MgCl_2$, 0.015 mM nitroblue tetrazolium and 8 nM 5-bromo-4-chloro-3-indoyl phosphate. The reaction was stopped by washing in 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA.

Rhodoquinone Depletion and Reconstitution of NADH-dependent Enoyl-CoA Reductase Activity in SMP—Mitochondria and SMP were prepared from adult *A. suum* as described previously, and rhodoquinone was extracted with dry pentane (5, 8, 14). SMP were lyophilized in 20 mM potassium phosphate, 2 mM HEPES-K^+, pH 7.5. After extraction with dry pentane, the SMP were resuspended (10 mg/ml) in 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, and 1 mM dithiothreitol. Rhodoquinone was prepared from adult *A. suum* muscle and SMP reconstituted as described by Sato *et al.* (26). NADH-dependent enoyl-CoA reductase was assayed as described previously (5). The complete assay system contained 20 mM potassium phosphate, pH 7.4, 175 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 50 μg of purified *A. suum* ETF and 2-methyl branched-chain enoyl-CoA reductase, 2 mg of *A. suum* SMP, 1 mM 2-methylcrotonyl-CoA, and 4 mM NADH. Preimmune and anti-ETF-RO IgG were prepared as described above and preincubated with SMP for 5 min prior to the initiation of the reaction. After incubation under nitrogen for 15 min, the reaction was terminated by the addition of 0.2 ml of 10% perchloric acid. 2-Methylcrotonyl-CoA and 2-MBCoA were determined in perchloric acid extracts by HPLC as described previously (27).

EPR Spectroscopy—The initial anaerobic reduction of ETF-RO was carried out as described by Beinert *et al.* (28). Oxygen was removed by repeated, successive applications of vacuum and argon. The purified ETF-RO was mixed with dithionite in a side arm of the apparatus, transferred to a quartz EPR tube, and frozen under argon in liquid nitrogen. The 9 GHz EPR measurements were performed using a Varian Century Line spectrometer equipped with a home-made, gas-phase liquid helium transfer line and quartz Dewar cavity insert. Temperature measurements were made using a 0.1 watt Allen Bradley carbon composition resistor calibrated at liquid helium, liquid nitrogen, and ambient temperatures. Frequency measurements were determined continuously using a Hewlett Packard 5340A frequency counter. Field calibration (>100 millitesla) was obtained at 20 K

using microwave powers that avoided modulation broadening. The scan time for each measurement was 4 min using a time constant of 0.128 s.

Potentiometry—The titration was carried out by a modification of the procedure of Wilson *et al.* (29) in the presence of the following mediators: dichlorophenolindophenol (+217 mV), phenazine methosulfate (+80 mV), phenazine ethosulfate (+55 mV), duroquinone (+7 mV), resorufin (−50 mV), 2-hydroxynaphthoquinone (−145 mV), phenosafranine (−239 mV), anthraquinone-2-sulfonic acid (−225 mV), benzylviologen (−311 mV), and methylviologen (−440 mV). The entire procedure was carried out in a glove bag provided with argon which was passed through a heated Ridox oxygen scrubbing system. The purified ETF-RO was deoxygenated by dialysis on ice in a continuously argon-purged apparatus that was inside the glove bag. The electrochemical measurements were made in a system consisting of a platinum disc working electrode and a silver/silver chloride reference electrode. The deoxygenated protein was placed in the electrochemical cell, and a stock solution of the mediators was added (6.2 mg of protein in 4 ml). Aliquots of a dithionite solution were added to the magnetically stirred protein-mediator solution in the cell. After each addition the electrochemical potential was allowed to come to equilibrium and recorded. An aliquot (300 μl) also was removed after each addition of dithionite and placed in an EPR tube. The tube was passed part way through a septum attached to the glove bag, and the sample was frozen in liquid nitrogen in a Dewar outside the bag. Once the sample was frozen, the EPR tube was removed through the septum from the bag. This procedure was repeated 11 times. The EPR spectrum for each sample was determined as previously described.

RESULTS

Purification of ETF-RO—A typical purification scheme for the adult *A. suum* muscle ETF-RO is presented in Table I. The enzyme is abundant in ascarid muscle mitochondrial membranes, and we estimate that it accounts for at least 2% of the total membrane protein. It is at least 10 times more abundant than the ETF-UO of porcine liver or beef heart mitochondria (9, 10). Because of its abundance and the simplified polypeptide composition of the ascarid mitochondria, the enzyme is essentially homogenous after DEAE-Bio-Gel chromatography, and the additional purification steps required for the pig liver enzyme usually were not necessary (10). The purified ETF-RO migrated as a single band during SDS-PAGE with an apparent molecular mass of 64.5 kDa (Fig. 1).

Properties of ETF-RO—The general properties of the ETF-RO are presented in Table II and are similar in many respects to the pig liver and beef heart ETF-UO, even though the ascarid enzyme functions in the oxidation of a low potential quinone, rhodoquinone, and the mammalian enzymes function in the reduction of a high potential quinone, ubiquinone. In fact, antisera to the beef heart ETF-UO readily recognized the ascarid ETF-RO on immunoblots of isolated ascarid mitochondrial membranes or purified preparations of the ETF-RO (Fig. 2). Similar results were obtained using antisera prepared against the ascarid enzyme and mitchondrial fractions from rat liver mitochondria (data not shown). The

TABLE I

Purification of ETF-rhodoquinone oxidoreductase from mitochondrial membranes isolated from adult A. suum body wall muscle

	Protein	Activity*	Specific activity	Recovery
	mg	*nmol/min*	*nmol/min/mg*	*%*
Membrane	522			
Cholate extraction	180	1260	7	100
Ammonium sulfate	22	678	31	53
DEAE Bio-Gel	5.2	586	113	47

*Nanomoles DPB reduced/minute.

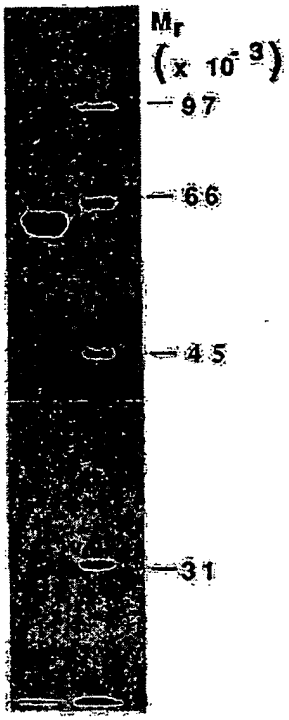

FIG. 1. SDS-polyacrylamide gel electrophoresis of the purified *A. suum* ETF-RO. The purified protein (10 μg) was separated by electrophoresis on a 10% SDS-polyacrylamide gel, as described under "Experimental Procedures." The gel was stained in 0.1% Coomassie Brilliant Blue in 7% acetic acid and destained in 7% acetic acid, 7% 2-propanol.

amino acid composition of the ETF-RO is summarized in Table III. The amino-terminal sequence of the purified protein is NVVNGKWITTHYTMH. This sequence is not similar to the predicted amino-terminal sequence of the human ETF-UO (30).

The visible absorption spectrum of the purified enzyme is presented in Fig. 3. The major absorption maxima were found at 272, 375, and 435 nm. The ETF-RO used in this study contained, per milligram of protein, 46.8 ng atoms of Fe, 47.6 ng atoms of acid labile sulfide, and 13.1 nmol of noncovalently bound flavin. Some preparations of ETF-RO exhibited increased absorbance between 410 and 420 nm. This increased absorbance presumably resulted from heme contamination and was removed by additional purification of the enzyme by chromatography on hydroxylapatite, as described under "Experimental Procedures."

EPR Spectroscopy and Potentiometry—The electron transfer properties of the ETF-RO were investigated by EPR spectroscopy. At 20 K, the EPR spectrum contained signals arising from an iron-sulfur center, $g = 2.076, 1.936, 1.883$, as well as a signal attributable to a flavin semiquinone radical (Fig. 4A). This flavin signal was more clearly detected in spectra recorded at 54 K or higher because at these temperatures the Fe/S signal was not detectable due to the lifetime broadening of the linewidths. The EPR spectrum obtained was consistent with a protein composed of both iron-sulfur and flavin centers. There was no evidence in the form of additional signals in the spectrum that might be interpreted as evidence for a direct interaction between the two spin systems.

An anaerobic potentiometric titration of the two signals in the EPR spectrum was carried out using a battery of redox

TABLE II

Comparison of properties of the A. suum ETF-RO and the mammalian ETF-UO

	Beef heart[a]	Pig liver[b]	*A. suum*[c]
Yield[d]	0.02%	0.03%	1.0%
M_r (subunit)	66,000	69,000	64,500
M_r (flavin)	74,000	73,000	68,000
Absorbance max (nm)	424	424	435
ESR spectrum			
gx	1.886	1.883	1.883
gy	1.939	1.939	1.936
gz	2.086	2.084	2.076
Fe:S:FAD	3.7:3.6:1	3.5:3.3:1	3.6:3.6:1
E_m (Fe:S)	−40 mV (pH 7.4)	−38 mV (pH 7.3) −47 mV (pH 7.5)[e]	−25 mV (pH 7.4)

[a] Ruzicka and Beinert (9).
[b] Beckman and Frerman (10).
[c] Present study.
[d] Milligram enzyme recovered/milligram protein.
[e] Paulsen *et al.* (34).

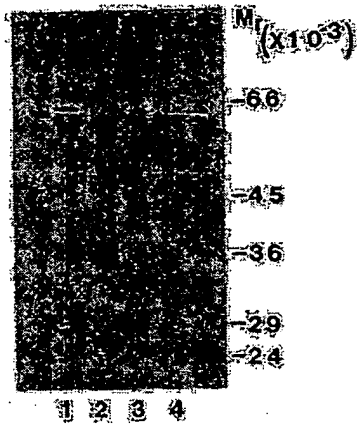

FIG. 2. Immunoblot of rat liver and *A. suum* mitochondria with antisera against the beef heart ETF-UO. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with antisera to the beef heart ETF-UO as described under "Experimental Procedures." *Lane 1*, rat liver mitochondria (40 μg); *lane 2*, *A. suum* mitochondrial supernatant fraction (40 μg); *lane 3*, *A. suum* mitochondrial membrane fraction (40 μg); *lane 4*, purified *A. suum* ETF-RO (2 μg).

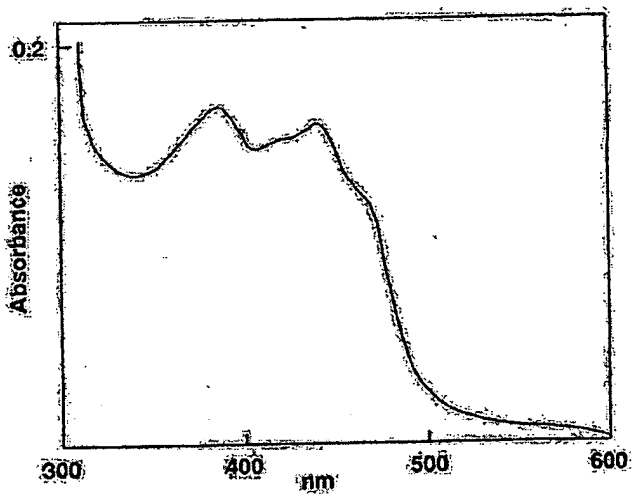

FIG. 3. Absorption spectrum of the purified *A. suum* ETF-RO. Spectra were recorded in 10 mM Tris·HCl, pH 7.4, 10% glycerol (w/v).

TABLE III

Amino acid analysis of the A. suum ETF:rhodoquinone oxidoreductase

Amino acid	*A. suum*	Pig liver[a]
Asx	58.0	59.6
Thr	29.9	32.9
Ser	33.2	28.1
Glx	61.2	68.0
Pro	30.6	43.4
Gly	77.6	65.7
Ala	52.8	41.2
Val	37.3	28.6
Met	7.9	12.9
Ileu	26.8	31.4
Leu	45.7	59.8
Tyr	17.3	17.2
Phe	13.6	24.0
His	35.4	23.8
Lys	48.6	42.6
Arg	31.0	27.9
1/2 Cys	ND[b]	8.8

[a] From Beckman and Frerman for ETF-UO from pig liver (10).
[b] ND, not determined.

mediators (Fig. 4*B*). The signal from the iron-sulfur center appeared upon reduction with a midpoint potential at +25 mV. The reduction of the flavin took place in two discrete steps. An EPR detectable flavin semiquinone anion radical was an intermediate in the two electron reduction of the cofactor. It was therefore possible to obtain reasonable estimates for the midpoint potential for both steps of the reduction of the flavin of +15 mV and −9 mV, respectively. The quantitative EPR *versus* potential data were fit to the Nernst equation ($n = 2$) using a non-linear least squares minimization strategy (Fig. 4*B*). It is not clear why the data were matched significantly better for $n = 2$ than for $n = 1$ in the Nernst equation simulations for this experiment, as has recently been reported for the pig liver ETF-UO (34). The quantitative EPR signals were consistent with the absorption of the same number of electrons by the iron-sulfur center as for each step in the reduction of the flavin, which is certainly a one-electron process. The observation is consistent with the protein behaving as a functional dimer under the conditions of this experiment. Whether this finding has relevance to the physiological condition remains to be determined. For example, the isolated ETF-RO may be associated in a way that is not possible in the mitochondrial membrane.

Quantitation of the EPR data yields a 48% recovery of the fully reduced iron-sulfur cluster when compared to the initial

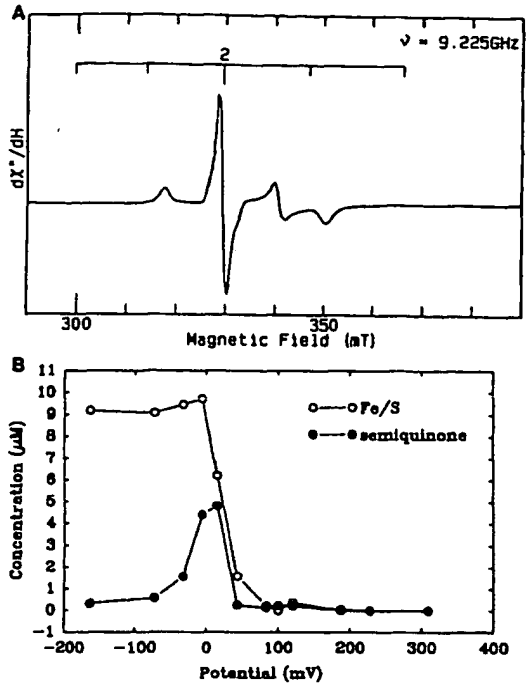

FIG. 4. **Electron paramagnetic resonance spectrum and potentiometric titration of the purified *A. suum* ETF-RO.** The purified *A. suum* ETF-RO was titrated anaerobically with dithionite in the presence of a number of redox mediators. Individual samples were transferred anaerobically to EPR tubes and frozen in liquid nitrogen. EPR measurements were performed as described under "Experimental Procedures." *Panel A*, EPR spectrum of the fully reduced ETF-RO recorded at 20 K; *panel B*, potentiometric titration of the *A. suum* ETF-RO.

concentration of ETF-RO estimated from total protein and apparent molecular weight from SDS-PAGE. This recovery is significantly lower than recoveries recently reported for the titration of the ETF-UO (34) and probably results in part from our inability to precisely determine the concentration of ETF-RO present at the beginning of the experiment. Molar absorptivity values, based on changes in visible spectra and quantitative amino acid analysis, are not available for the ETF-RO. The low recovery in the present study is unlikely to be due to the presence of ETF-RO missing the FE:S cluster given the good correlation between flavin, iron, and sulfide observed. In addition, analysis of the EPR spectra at different states of reduction gives no indication of a conversion of a portion of the cluster to an S = 3/2 state or for spin-spin interactions between the reduced cluster and the flavin semiquinone.

Quinone Dependence and Role of ETF-RO in NADH-dependent Enoyl-CoA Reduction—Isolated *A. suum* adult muscle SMP were incubated under nitrogen with the purified *A. suum* ETF and 2-methyl branched-chain enoyl-CoA reductase, NADH and 2-methylcrotonyl-CoA, and the formation of 2-MBCoA was determined (Table IV). 2-MBCoA formation was dependent on the presence of NADH, ETF, and the 2-methyl branched-chain enoyl-CoA reductase and was inhibited by rotenone at concentrations observed to inhibit electron-transport in *A. suum* muscle SMP (10). More importantly, IgG against the ascarid ETF-RO inhibited 2-MBCoA formation, while preimmune IgG had no effect, attesting to the importance of the ETF-RO in this pathway. Quinone extraction of lyophilized *A. suum* muscle SMP with dry pentane resulted in a significant loss (>90%) of NADH-dependent enoyl-CoA

TABLE IV

Quinone dependence of NADH-dependent 2-methylcrotonyl-CoA reduction in submitochondrial particles isolated from adult A. suum muscle mitochondria

The complete assay system contained 20 mM potassium phosphate, pH 7.4, 175 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 50 μg of purified *A. suum* ETF, 50 μg of purified *A. suum* 2-methyl branched-chain enoyl-CoA reductase, 3 mg of *A. suum* submitochondrial particles, 1 mM 2-methylcrotonyl-CoA, and 4 mM NADH in a final volume of 1 ml. After incubation for 15 min under nitrogen, the reaction was terminated by the addition of 0.2 ml of 10% perchloric acid. Pentane extraction and addition of quinones was accomplished as described under "Experimental Procedures." In experiment 2, SMP were preincubated for 5 min with IgG prior (50 μg) to the initiation of the reaction. CoA esters were determined by HPLC.

Assay Additions	2-Methycrotonyl-CoA	2-Methylbutyryl-CoA
	nmol	
Experiment 1		
SMP	475	391
+ rotenone	848	25
SMP (pentane extracted)		
+ rhodoquinone	555	193
+ ubiquinone	744	42
Experiment 2		
SMP	463	373
+ preimmune sera	495	340
+ anti ETF:RO	552	180

reductase activity (Table IV). The reincorporation of rhodoquinone, previously extracted from *A. suum* muscle mitochondria, restored over 50% of the NADH-dependent reductase activity in the reconstituted system. In contrast, the incorporation of ubiquinone was ineffective in restoring 2-MBCoA formation. A similar quinone dependence has been observed for the reconstitution of NADH-dependent fumarate reductase activity in SMP from adult *A. suum* muscle (14, 33).

DISCUSSION

An ETF-RO has been purified to apparent homogeneity from the anaerobic mitochondria found in the body wall muscle of the adult parasitic nematode, *A. suum*. These organelles catalyze a reversal of β-oxidation which results in the accumulation of unique branched-chain fatty acids as end products of carbohydrate metabolism (2, 6). The ETF-RO participates in the final reaction in this pathway, the NADH-dependent reduction of 2-methyl branched chain enoyl-CoAs, as outlined in Fig. 5. The ascarid ETF-RO is more abundant than the corresponding ETF-UO of aerobic, mammalian mitochondria, attesting to the importance of branched-chain fatty acid synthesis in the ascarid organelle. In fact, branched-chain fatty acids accumulate to over 100 mM in *A. suum* perienteric fluid and rival Cl^- as the major extracellular anions (31). Surprisingly, the ascarid and mammalian enzymes are quite similar in many respects, even though they function in opposite directions *in vivo*. The ascarid ETF-RO shuttles reducing equivalents from the unique low potential quinone, rhodoquinone (E_m = −64 mV) to a soluble ETF, while the mammalian ETF-UO transfers electrons from ETF to ubiquinone (E_m = +110 mV) of the electron-transport chain.

In mammalian mitochondria, each step in the pathway of β-oxidation appears to be reversible under physiological conditions and the reverse electron flow from pig liver ETF-UO to ETF has been extensively characterized (11). In fact, Frerman has suggested that the ratio of oxidized to reduced ETF depends on the mitochondrial NADH/NAD^+ ratio and that the overall rate of β-oxidation may be regulated in a similar manner (11). Other organisms are capable of catalyz-

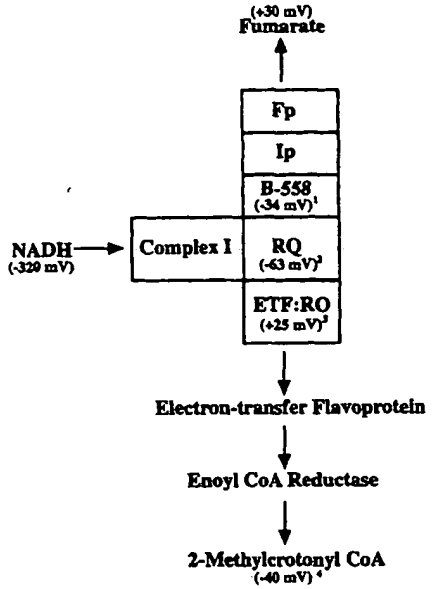

FIG. 5. **Pathway of NADH-dependent 2-methyl branched-chain enoyl-CoA reduction in muscle mitochondria of adult *A. suum*.** F_p, flavoprotein of fumarate reductase (2, 27); I_p, iron-sulfur protein of fumarate reductase (2, 33). Source of redox potentials, 1 and 2, Kita *et al.* (33); 3, present study; 4, Lenn *et al.* (38).

ing an NADH-dependent enoyl-CoA reduction, but *A. suum* is unique in that its electron-transport chain contains a low potential quinone and enoyl-CoA reduction appears to be coupled to rotenone-sensitive electron-transport associated energy generation (5, 32). The present study demonstrates that ubiquinone cannot replace rhodoquinone in this NADH-dependent reduction, as has been observed previously in these organelles for the energy-linked NADH-dependent reduction of fumarate to succinate (14, 33).

The calculated redox potentials of the iron-sulfur center and the two steps in the complete reduction of the the flavin of the ascarid ETF-RO are +25 mV, +15 mV and −9 mv, respectively, at pH 7.4. Recently, similar values of +47 mV, +28 mV, and −6 mV have been reported for the pig liver ETF-UO, especially given the marked pH dependence of the midpoint potential of the mammalian enzyme (10, 34). The redox potential of the different components of the *A. suum* electron-transport chain are illustrated in Fig. 5. Reported redox potentials for the enoyl-CoA/acyl-CoA couple vary greatly (35, 37). Recently, Lenn *et al.* (38) have convincingly demonstrated that acyl-CoAs are essentially isopotential for chain lengths of C-4 to C-16 (E_m = −40 mV), and values for 2-methyl branched chain derivatives are probably similar to their straight-chain counterparts. Interestingly, dramatic shifts in the midpoint potentials of both the *Megasphaera elsdenii* butyryl-CoA dehydrogenase and the pig kidney general acyl-CoA dehydrogenase have been observed when substrate binds (38, 39). This may create a more thermodynamically favorable transfer of electrons from substrate to enzyme (38–39). Based on the potentials outlined in Fig. 5, it is clear that the iron-sulfur center of the ascarid ETF-RO would be readily reduced under physiological conditions, but its reoxidation would be more problematic. The redox potential of the ascarid ETF-RO is more positive than either rhodoquinone or the enoyl-CoA/acyl-CoA couple, suggesting that its reoxidation may be the rate-limiting component of the pathway. Interestingly, enoyl-CoA/acyl-CoA ratios are dramatically elevated in isolated ascarid mitochondria and presumably facilitate the reoxidation of ETF-RO through mass action (27). In fact, little acyl-CoA accumulates in ascarid mitochondria and they possess an active CoA transferase specific for 2-methyl branched-chain acyl-CoAs.[2] The redox potential of the ETF-RO is also much more positive than the *b* cytochrome (E_m = −34 mV) associated with the fumarate reductase, suggesting that ETF-RO should be able to effectively compete for electrons from reduced rhodoquinone (33). However, since, the fumarate/succinate couple has a potential of +30 mV, the *b* cytochrome should be readily reoxidized, especially at high fumarate to succcinate ratios. This may explain why fumarate dramatically inhibits NADH-dependent 2-methyl branched-chain enoyl-CoA reduction in a reconstituted ascarid system (8). Apparently, 2-methyl branched-chain enoyl-CoA reduction serves as a sink for excess reducing power when fumarate reduction is limited. Predictably the ETF-RO should be in a much more reduced state than the fumarate reductase *in vivo*, although this hypothesis has never been tested directly.

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